# Cellular Thermogenic Mechanisms

## A Comparative Study of Brown Adipose Tissue and Skeletal Muscle.

by

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# Abstract


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Thermogenic mechanisms in homeotherms appear to be largely controlled by norepinephrine and the sympathetic nervous system. The thermogenic mechanism of brown adipose tissue (BAT) has been well characterized by other researchers and recent data from our laboratory have suggested a role for the vasculature in skeletal muscle thermogenesis. The aim of this thesis was to compare the thermogenic mechanisms of BAT and skeletal muscle. The contribution of the vasculature to BAT thermogenesis and the possibility of a mitochondrial uncoupling mechanism in skeletal muscle were investigated.

To examine the vascular contribution to BAT thermogenesis a technique for the isolation and constant flow perfusion of the periaortic BAT deposit was developed and characterized. In this preparation, the thermogenic response was found to be entirely β-adrenoreceptor mediated. Similar increases in oxygen consumption were induced by norepinephrine, isoproterenol and BRL 35135A (a specific $\beta_3$-agonist). These induced responses were completely inhibited by propranolol, but unaffected by either phentolamine or nitroprusside (a nitrovasodilator). The lack of an α-mediated or nitroprusside-sensitive effect suggested that the vasculature did not contribute significantly either directly (e.g. "hot pipes") or indirectly (through alterations in blood flow) to BAT thermogenesis.

Initially, perfused rat hindlimbs were used in our laboratory to address the question of uncoupling in skeletal muscle. When infused the rate of conversion of a redox dye (MTT) was decreased by the addition of norepinephrine in association with a stimulation of oxygen consumption. Similarly, measurement of muscle cell mitochondrial membrane potential, using [$^3$H]-TPMP, suggested decreases were occurring in the presence of norepinephrine. Together, these data implied that norepinephrine could be causing uncoupling in skeletal muscle mitochondria. Thus, a search for the mechanism responsible for this thermogenic process was conducted using isolated subsarcolemmal skeletal muscle mitochondria.

Potential candidates that led to increased oxygen consumption and decreased MTT conversion by subsarcolemmal skeletal muscle mitochondria were assessed in conjunction with measurements of mitochondrial membrane potential (using Rhodamine 123). Mitochondria exhibited little respiratory control when succinate was used as the substrate, suggesting that it may be able to act as an uncoupler. With

succinate as the substrate, respiration was maximal despite an inhibition of the rate of MTT conversion. The inhibition of MTT conversion was overcome by the transition of the mitochondria from state IV to state III, and this ADP-regulated, succinate-induced uncoupling appears specific for skeletal muscle mitochondria. Although succinate seems a viable candidate for the uncoupling seen in skeletal muscle, it does not decrease the mitochondrial membrane potential and this eliminates it as the uncoupler which is acting in the perfused rat hindlimb.

As glycerol release has been noted from the perfused rat hindlimb on addition of norepinephrine, fatty acids and their uncoupling action were investigated in the isolated mitochondria. Fatty acids gave responses with isolated mitochondria similar to that seen on the addition of uncouplers such as FCCP. Respiration was increased, while both membrane potential and MTT conversion were decreased.

In conclusion, the development and examination of a novel perfused BAT preparation suggested that the vasculature does not contribute significantly to thermogenesis in a manner similar to that seen in skeletal muscle. In skeletal muscle, however, fatty acids are a candidate for an uncoupler that can act in a manner similar to that seen in BAT. With other researchers having shown that the induced loss of mitochondrial membrane potential can be prevented if ADP and $Mg^{2+}$ are present, fatty acids may be able to induce uncoupling that is readily reversed with the onset of exercise.

# Preface

The experimental work presented in this thesis was performed between February 1991 and January 1995 in the Biochemistry Department at the University of Tasmania. All experiments were approved by the Ethics Committee of the University of Tasmania under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990). Work performed by other members of this department has been presented and acknowledged where relevant. The majority of this is in Chapter 3 where data from hindlimb studies has been presented as the establishment of the criteria needed for an uncoupler of skeletal muscle mitochondria.

The duckling experiments were conducted in the Laboratoire de Thermorégulation et Energétique de l'Exercise at the Universite Claude Bernard Lyon, Lyon, France during a three week visit in October 1994.

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Final thanks must go to my parents for putting up with me all these years and for their support. It was all greatly appreciated.

# Contents

# Abbreviations used in this thesis

| | |
|---|---|
| ADP | adenosine diphosphate |
| AMP | adenosine monophosphate |
| AMP-PCP | adenylylmethylenediphosphate |
| AMP-PNP | 5' adenylylimidodiphosphate |
| ANT | adenine nucleotide translocator |
| ATP | adenosine triphosphate |
| ATP-γ-S | adenosine 5'-O-(-3-thiotriphosphate) |
| BAT | brown adipose tissue |
| BSA | bovine serum albumin |
| CA | cold acclimated |
| COX | cytochrome oxidase |
| EM | electron microscope |
| FCCP | carbonyl cyanide *p*-trifluoromethylphenylhydrazone |
| FFA | free fatty acid (non-esterified) |
| GDP | guanosine diphosphate |
| GTP | guanosine triphosphate |
| GUI | glucose utilization index |
| HK | hexokinase |
| IMF | intermyofibrillar |
| ISO | isoproterenol |
| LDH | lactate dehydrogenase |
| MTT | [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] |
| NE | norepinephrine |
| OD | optical density |
| PCP | pentachlorophenol |
| PFK | phosphofructokinase |
| RCR | respiratory control ratio |
| Rh123 | Rhodamine 123 |
| SKM | skeletal muscle |
| SS | subsarcolemmal |
| TN | thermoneutral |
| [$^3$H]-TPMP | [$^3$H]-triphenylmethylphosphonium |
| $\dot{V}O_2$ | rate of oxygen uptake |
| WAT | white adipose tissue |

# List of Tables

# List of Figures

# Chapter 1

## General Introduction:
## Contemporary views on the mechanism of non-shivering thermogenesis

### 1.1 *Introduction*

Homeothermy is defined as the maintenance of a constant body temperature irrespective of that of the environment. Changes in environmental temperatures - both diurnal and seasonal - present a challenge that homeothermic species have developed a number of different tactics to cope with (eg. hibernation, acclimatization, torpor). Despite these varying responses, all at some point in their life cope by increasing their heat production (Horwitz, 1989). The regulation of metabolism and the tissues involved in producing the extra heat required under colder environmental conditions still requires elucidation.

### 1.2 *Thermogenesis*

Thermogenesis is simply defined as the production of heat (Himms-Hagen, 1976). Heat is produced as a product of all metabolic reactions and can be classified under two major headings - obligatory and facultative.

#### 1.2.1 *Obligatory thermogenesis*

The heat generated by those metabolic reactions that are necessary to maintain cell integrity and the steady state condition of the animal are termed obligatory (Himms-Hagen, 1989; Horwitz, 1989). These include the processing of food during ingestion, digestion, absorption and subsequent storage. All tissues contribute to obligatory thermogenesis and this form of heat production is possessed by both heterotherms and homeotherms.

#### 1.2.2 *Facultative thermogenesis*

Upon exposure to a cold environment, homeotherms require the production of additional heat to maintain a constant body temperature (Dawkins and Hull, 1965). For small homeothermic animals, with a large surface to volume ratio, temperature regulation becomes difficult with losses to the environment exceeding normal metabolic heat production. Human neonates are amongst the many small homeotherms that face this problem at birth (Aherne and Hull, 1966; Cannon and

Nedergaard, 1982). Maintenance of a constant body temperature is therefore achieved by the initiation of regulatory thermogenic mechanisms (Trayhurn, 1979; James and Trayhurn, 1981) and the ability to initiate these processes is the prime prerequisite for homeothermy.

Facultative thermogenesis consists of both voluntary and involuntary processes that are able to be activated and deactivated relatively rapidly, usually by the nervous system. The main effectors of this form of thermogenesis are considered to be skeletal muscle and brown adipose tissue, although other tissues have also been suggested to be involved. Skeletal muscle is considered to be involved in both voluntary exercise-induced thermogenesis and involuntary cold-induced shivering that is controlled by the central nervous system via the motor nerves to the muscle. Brown adipose tissue (BAT) thermogenesis is regulated by the sympathetic nervous system via autonomic nerves which directly innervate the BAT cells. Increased sympathetic nerve activity is induced by both cold exposure (non-shivering thermogenesis) and dietary intake (diet-induced thermogenesis) (Himms-Hagen, 1989).

### 1.3 *Brown adipose tissue*

All tissues produce heat as a byproduct of normal metabolic processes but only one organ, BAT, appears to have heat as the primary product. The thermogenic activity of BAT, its contribution to the overall metablic rate and its necessity to a homeotherm is the subject of much controversy.

Since it was first described by the Swiss naturalist Konrad von Gesner more than 400 years ago, BAT has been studied extensively in an attempt to discover its origins, its uses, its mode of action, and its species distribution (Foster, 1986). Because of its involvement with thermoregulation, especially with the problems associated with survival in cold environments and its seeming role in the regulation of obesity, BAT has been of interest for many years.

The thermogenic activity and thus contribution of BAT to the metabolic rate of a mammal is dependent upon the amount of tissue present, its mass-specific thermogenic capacity and the degree to which it is stimulated by the sympathetic nervous system or by exogenous catecholamines in an experimental situation (Foster, 1986). This varies between species and with physiological and other circumstances. The contribution of BAT can be 1% or less for example at thermoneutrality, or when stimulated by cold or overeating, this contribution has been estimated to be as great as 50% of the overall metabolic rate. A contribution of this magnitude is remarkable, in that BAT rarely, if ever, amounts to more than a few percent of the total body mass

(0.5 - 2.0% is common in rodents; 3 - 6% in some hibernators and neonates - Foster, 1986). In its anatomical distribution, its histological appearance and its function BAT is distinct from white adipose tissue (WAT; Heaton, 1972).

### 1.3.1 *Anatomical distribution*

BAT occurs as discrete lobes that are invaginated by thin strands of connective tissue, while WAT is distributed in a diffuse state throughout the body (Arbuthnott, 1989). The anatomical distribution (Table 1.1) is in fact strategic, with BAT generally found in immediate contact with the main blood vessels, overlying the venous drainage from the periphery of the body and the major organs, neuronal elements and vessels within the body (Nechad, 1986). Depending on the acclimation and nutritional status of an individual, and the species, the relative proportions of total BAT in these disparate locations varies (Himms-Hagen, 1989), as demonstrated by Foster and his colleagues (1980) in warm- and cold-acclimated rats (Table 1.1).

**Table 1.1** Anatomical locations of BAT deposits and the proportion of the total amount of BAT in the body that is located at each site in warm-acclimated and cold-acclimated rats. (Adapted from Foster *et al.*, 1980).

| Deposit | Warm-acclimated | Cold-acclimated |
|---|---|---|
| Interscapular | 20 % | 23 % |
| Periaortic | 25 % | 25 % |
| Perirenal | 26 % | 24 % |
| Dorsal cervical | 3 % | 3 % |
| Axillary | 18 % | 16 % |
| Iliac and Inguinal | 8 % | 9 % |
| Total | 100 % | 100 % |

### 1.3.2 *Comparison of brown and white adipose tissues*

Histologically, BAT is a more vascular tissue than WAT, being supplied with a rich capillary nexus. This additional vascularization, together with the presence of large amounts of mitochondrial cytochromes, gives BAT its distinctive reddish-brown colour. WAT, in contrast, is yellow or white. BAT is also highly innervated by postganglionic fibres (Arbuthnott, 1989), with nerve endings present on both the adipocytes and the cells of the vascular network. The sympathetic innervation to WAT is limited to the blood vessels (Himms-Hagen and Phil, 1984).

These two fat types also differ in their cellular makeup, due to their functional differences. As WAT is basically a storage organ, it is literally composed of a large lipid droplet (Arbuthnott, 1989) with little cytosol whereas BAT, with its primary function being the production of heat, contains many small lipid droplets (Fain and Garcia-Sainz, 1983; Girardier, 1983). These two tissues also differ enzymatically as seen in Table 1.2, with BAT being the more active tissue.

**Table 1.2** Maximal activities of some key enzymes of carbohydrate, ketone body, adenosine and glutamine metabolism in white and brown adipose tissue. Results are means ± SEM for at least six animals. (Cooney *et al.*, 1986).

| Enzyme | Enzyme activities (nmol/min per mg protein) at 25°C | |
|---|---|---|
| | White adipose tissue | Brown adipose tissue |
| Glucokinase | < 0.1 | < 0.1 |
| Hexokinase | 31.9 ± 2.61 | 61.6 ± 5.5 |
| Glucose 6-phosphatase | < 0.1 | < 0.1 |
| 6-Phosphofructokinase | 41.8 ± 5.80 | 67.0 ± 3.0 |
| Fructose bisphosphatase | < 0.1 | < 0.1 |
| Pyruvate kinase | 90.8 ± 8.87 | 403 ± 4.51 |
| Pyruvate carboxylase | 19.6 ± 0.49 | 36.8 ± 0.62 |
| Phosphoenolpyruvate carboxykinase | 4.6 ± 0.44 | 13.1 ± 0.35 |
| Glucose 6-phosphate dehydrogenase | 2.2 ± 0.31 | 31.9 ± 1.10 |
| 3-Hydroxybutyrate dehydrogenase | 2.1 ± 0.25 | < 0.1 |
| 3-Oxoacid CoA dehydrogenase | 21.1 ± 0.85 | 204 ± 42 |
| Acetoacetyl-CoA thiolase | 18.1 ± 0.32 | 182 ± 38 |
| 5'-Nucleotidase | 11.4 ± 1.31 | 3.2 ± 0.35 |
| Adenosine deaminase | 18.1 ± 1.81 | 14.2 ± 0.57 |
| Adenosine kinase | 0.27 ± 0.04 | 0.09 ± 0.01 |
| Citrate synthase | 38.2 ± 3.06 | 380 ± 13.7 |
| Glutaminase | 24.9 ± 0.8 | 39.0 ± 3.2 |

The predominant histological difference between brown and white adipocytes besides that of their lipid division status, is in the size (Afzelius, 1970) and number (Nedergaard and Lindberg, 1982) of mitochondria that are present in the cytosol. Mitochondria from brown adipocytes are larger (greater than 0.5μm compared to less than 0.3μm), more numerous, and their cristae are typically tightly packed (Fain and

Garcia-Sainz, 1983) and cross the whole width of the organelle compared to those present in white adipocytes. The mitochondrial inner membrane surface area is greater than the outer (Lindgren and Barnard, 1972) and it differs structurally from that found in other tissues in that it lacks elementary particles (Lindberg *et al.*, 1967). BAT mitochondria also possess a unique proton conductance pathway that appears to be directly related to its role in heat production.

### 1.3.2.1 *BAT uncoupling protein*

This conductance pathway creates a regulatable alternative pathway for proton re-entry into the mitochondrial matrix (Nicholls, 1974). It facilitates the return through the inner membrane of $H^+$ which are generated from substrate oxidation through the respiratory chain. This allows ATP synthesis to be bypassed and the energy generated by substrate oxidation to be expressed as heat (Klingenberg, 1990; Klaus *et al.*, 1991). The BAT uncoupling protein is the only carrier known that can transport $H^+$ alone and this was first demonstrated (Nicholls, 1976b) in swelling experiments with appropriate ion combinations in isolated mitochondria.

Nicholls (1976a,b) summarized the then current knowledge of this BAT mitochondrial conductance pathway. At that time, it was known that the conductance pathway was inhibited by the presence of exogenous, not matrix, purine nucleoside di- or triphosphates and that the binding site for this inhibition was on the outside of the mitochondrial inner membrane. This binding site had been shown to be distinct from the adenine nucleotide translocator, to not bind atractylate, and to bind purine nucleotides with pH-dependent affinities. No similar binding site had been detected in rat liver mitochondria.

About the same time, Ricquier and Kader (1976) reported an increase in the content of an unknown polypeptide (of apparent molecular weight 32000 daltons) in BAT after cold adaptation. They found that this upregulation was reversed during re-adaptation to a normal housing temperature of 22°C and that this unknown protein was located in the pellet or membrane fraction, not the supernatant fraction, of KCl treated mitochondria. This protein was found to be the only one that was significantly altered with cold adaptation when calculated as a percentage of total mitochondrial protein. Two proteins of apparent molecular weight 30000 and 32000 (representing 6% and 10% of the inner membrane protein respectively) were identified by Heaton *et al.* (1978) using 8-azido-adenosine [$\gamma$-$^{32}$P] triphosphate covalently bound to hamster brown adipose tissue mitochondria by near-ultraviolet irradiation. Selective labelling allowed identification of the 30000-Mr protein as the carboxyatractylate binding component of the adenine nucleotide translocator and the 32000-Mr as the regulatory site of the energy dissipating ion uniport. As shown by Ricquier and Kader (1976) in

the rat, Heaton *et al.* (1978) also found that the expression of this protein increased in cold-acclimated guinea pigs.

In 1983, Rial and colleagues (Rial *et al.*, 1983) reported that the increased proton permeability induced by the presence of fatty acids to non-respiring hamster BAT mitochondria was inhibited by the addition of purine nucleotides. Purine nucleotides do not affect the increases in proton permeability induced by the addition of synthetic proton translocators such as carbonylcyanide-p-trifluoromethoxy phenylhydrazone (FCCP). This data indicates that the uncoupling effect of the free fatty acids is via interaction with the 32000-Mr protein. They also reported that although the fatty acids were able to lower the affinity of nucleotide binding to the 32000-Mr protein, their uncoupling action was not due to the displacement of purine nucleotides as this effect was relatively minor. They further showed that the modulation of proton permeability in BAT mitochondria by fatty acids was dependent on chain length, extent of unsaturation, the presence of an unesterified carboxyl group and pH.

Rial and Nicholls (1984) assessed the function of the uncoupling protein in terms of its effective proton conductance and, as proposed by Locke *et al.* (1982a,b), the possibility that fatty acids are the physiological regulators of the uncoupling protein. They showed that during cold adaptation the correlation between the presence of the uncoupling protein and the ability of long-chain fatty acids to override the nucleotide-induced low-conductance state held, but that mitochondria from the newborn guinea pig were anomalous. These mitochondria were found to have a high GDP titre and nucleotide-sensitive conductance, but a low sensitivity to fatty acids and an anomalously high basal proton conductance. A similar, although smaller anomaly was also found in the 17 h-cold stressed animals, with an enhanced basal conductance and a decreased sensitivity to fatty acids relative to the warm controls. At birth and during the first few hours of cold stress, when the thermogenic demand on BAT is at its most intense, these variations from the expected behaviour could be very significant.

The uncoupling protein is not a channel but behaves kinetically like carriers for other solutes. Being a carrier rather than a channel, the uncoupling protein does not cause a total dissipation of the membrane potential of the BAT mitochondria. This presence of a reasonable membrane potential, despite the regulated influx of the protons, is obligatory for the maintenance of a low level of ATP synthesis and for driving other energy-dependent homeostatic processes (Klingenberg, 1990).

Loncar (1990) found that the uncoupling protein is located in the cristae of BAT mitochondria and not in those of other tissues (*e.g.* endothelium, fibrocytes, smooth muscle cells, Schwann cells, axons of neural cells and white blood cells). This

protein is probably one of the mitochondrial proteins synthesized by the ribosomes which are in contact with the outer mitochondrial membrane (these regions contain clusters of uncoupling protein moleculaes and clusters of RNase-gold particles which indicate active protein synthesis). It is then either extruded into the intermembranous space or directed by lateral movement into intermembranous contact sites and incorporated into the inner mitochondrial membrane (Loncar, 1990).

Klaus *et al.* (1991) and references therein is a recent review on the current knowledge of the uncoupling protein. The uncoupling protein functions as a dimer, with only one nucleotide binding site per dimer, and infra red spectroscopic studies suggest that the secondary structure consists of a 50% α-helix, 28-30% β-structure, 13-15% β-turn and 7% unordered distribution. The amino acid sequence has been determined for hamster, rat, mouse, calf, rabbit and human, and the sequences from these different species are all very similar. For example, human and rat are 79% identical. Regulation of gene expression and the structure-function relationships of the protein itself still require further investigation.

This uncoupling protein has been demonstrated to be exclusively located in brown adipocyte mitochondria (Cannon *et al.*, 1982; Lean and James, 1983).

### 1.3.3 *BAT thermogenesis*

Thermogenesis in BAT is regulated by sympathetic innervation (Himms-Hagen and Phil, 1984; Shimazu, 1984) with the hypothalamus appearing to exert direct control over this action (Himms-Hagen *et al.*, 1989; Gong *et al.*, 1990). Release of the thermogenic effector norepinephrine is usually prompted by stimulation of the ventromedial region of the hypothalamus (Perkins *et al.*, 1981; Minokoshi *et al.*, 1986; Freeman and Wellman, 1987; Saito *et al.*, 1987; Sakaguchi and Bray, 1987; Holt *et al.*, 1987, 1988) although stimulation of the paraventricular nucleus (Freeman and Wellman, 1987; Holt *et al.*, 1987, 1988) can also lead to BAT thermogenesis. Other areas of the brain are also involved in the regulation of BAT thermogenesis but they have not as yet been pinpointed (Himms-Hagen, 1989).

Release of norepinephrine from the sympathetic nerve endings initiates thermogenesis in brown adipocytes via interaction with an atypical β-adrenoreceptor (see Section 1.3.3.1). This stimulates adenylate cyclase with a resulting increase in cyclic-AMP production that in turn causes protein kinase-dependent activation of a triglyceride lipase, accelerating lipolysis. The fatty acids that are then produced are a fuel for the resulting thermogenesis and have been suggested to act as an intracellular signal for the operation of the proton-conductance mechanism in the mitochondria (Himms-Hagen and Phil, 1984; Nicholls *et al.*, 1986; Himms-Hagen, 1989; Trayhurn, 1989). Purine nucleotides (ATP, ADP, GTP and GDP) have been shown to cause

recoupling of BAT mitochondria by binding to the uncoupling protein and thereby inhibiting the proton short circuit (as reviewed by Nicholls and Locke, 1984 and Trayhurn, 1989 and references therein).

In mitochondria, respiration leads to the formation of a proton gradient across the inner mitochondrial membrane. Normally, proton reentry into the mitochondrial matrix is regulated via the $F_0F_1$-ATPase. As the protons pass through this protein, ADP is phosphorylated to form ATP. The BAT uncoupling protein allows regulated reentry of the protons without the necessity for ADP phosphorylation. Thus energy is released as heat rather than trapped in the ATP molecule.

#### 1.3.3.1 *Atypical BAT β-adrenoreceptor*

The adrenoreceptor responsible for BAT thermogenesis was initially thought to be one of the known β-adrenoreceptors (Bukowiecki *et al.*, 1980). In isolated brown adipocytes, the β-agonist isoproterenol was found to stimulate oxygen consumption to the same extent as that of the mixed agonist norepinephrine and the stimulated thermogenesis was inhibited by the β-antagonist propranolol, not by the α-antagonists phentolamine and phenoxybenzamine. In fact, the affinity sequence for the physiological activity of agonists corresponded to that of typical $\beta_1$-adrenoreceptors: isoproterenol >> norepinephrine ≈ epinephrine >> phenylephrine (Bukowiecki *et al.*, 1980 and references therein).

With the development of a novel group of β-agonists, it was found that the BAT adrenoreceptors differed from that of the known $\beta_1$ and $\beta_2$ subtypes (Arch *et al.*, 1984; Arch and Kaumann, 1993). These agonists were able to selectively stimulate lipolysis in brown adipocytes but were less potent as stimulants of atrial rate or tracheal relaxation (where $\beta_1$ and $\beta_2$ subtypes predominate respectively). In contrast to this, isoproterenol, fenoterol and salbutamol were more potent stimulators of atrial rate and tracheal relaxation than of BAT lipolysis (Arch *et al.*, 1984). Although β-antagonists (propranolol, atenolol, ICI 118,551 and pindolol) are able to inhibit the thermogenesis induced in brown adipocytes by either isoproterenol or one of the newer, more specific agonists (BRL 28410, BRL 37344 or CGP-12177A), their low pA2 values are a further indication that this receptor is atypical (Arch, 1989; Scarpace and Matheny, 1991). *In vivo* behavioural studies (Carlisle and Stock, 1991) using various β-agonists again affirmed the existence of this atypical adrenoreceptor. In this latter study, it was found that isoproterenol caused small increases in colonic temperature, large increases in the demand for exogenous heat and profound and lethal hypothermia. In contrast to this, the use of a novel selective agonist (RO40-2148) produced larger increases in colonic temperature and decreased demand for

exogenous heat (Carlisle and Stock, 1991). This atypical receptor has become known as the $\beta_3$-adrenoreceptor.

This adrenoreceptor has been found to be abundantly expressed only in brown and white adipose tissues (Granneman *et al.*, 1991; Granneman and Lahners, 1992; Muzzin *et al.*, 1991) although they may also be present on tracheal epithelium (Webber and Stock, 1992), bronchial smooth muscle (Tamaoki *et al.*, 1993) and skeletal muscle membranes (Sillence *et al.*, 1993). Most commonly studied animal species - rat, hamster, rabbit and dog - possess this adrenoreceptor (Langin *et al.*, 1991; Galitzky *et al.*, 1993; Arch and Kaumann, 1993 and references therein) as do humans (Emorine *et al.*, 1992). In humans, it has been found to be expressed in the gallbladder, colon and adipose tissue, but not in skeletal muscle, heart, liver, kidney, thyroid or lymphocytes (Krief *et al.*, 1993; Emorine *et al.*, 1994).

### 1.3.4 *Development and age distribution of BAT*

In the newborn or young of many eutherian species, BAT is particularly prominant and is located in most if not all of the deposits mentioned in Table 1.1 (Himms-Hagen, 1983, 1989). It is the means whereby they are able to compensate for the heat loss encountered at birth on leaving a protected and thermoneutral environment (Nechad, 1986; Nedergaard and Cannon, 1992). This fall in environmental temperature is approximately 12-15°C for most humans, and 30°C or more for some animals in the field (Laburn *et al.*, 1994). As this challenge is seldom lethal, it indicates that neonates possess an adequate thermoregulatory competence at birth.

The development of BAT begins *in utero*, although in some eutherians (hamsters) or in some particular areas (thoraco-periaortal and medio-perirenal areas in rats) the development of thermogenic adipose tissue begins after birth (Houstek *et al.*, 1990). In smaller eutherians, postnatal BAT development is characterized by quantitative changes in the number of mitochondria, the amount of uncoupling protein and the amount of lipid that is present (Loncar, 1991a). Although many eutherians possess BAT at birth, the amount varies considerably from species to species (Dawkins and Hull, 1965). In adults of many species, BAT appears to have been converted to white fat, but the age at which these postnatal BAT changes occur varies between species. Within the first postnatal month, cats, rabbits and sheep undergo changes in many BAT deposits (including the interscapular and inguinal deposits) that change the adipocytes from a thermogenic to a lipid storage form, while in humans, this same process can take up to 15-20 years (Loncar, 1991a). Rats, too, possess large amounts of BAT, but unlike some species, they retain significant amounts in all reported locations in the adult.

As eutherians age, the heat lost per unit body weight decreases as a result of improved insulation and increased body size (or decreased surface to volume ratio). In smaller eutherians, although insulation may improve, the surface to volume ratio does not decrease markedly and this results in the continued stimulation of the tissue. Because of this, BAT is macroscopically identifiable throughout life in smaller eutherians.

In larger eutherians (eg. humans, cats and dogs) where the surface to volume ratio decreases and insulation increases, BAT deposits are transformed into white-fat-like tissues. In many BAT deposits lipids accumulate, the adipocytes become unilocular, the tissues vascularization and innervation decreases, intercellular connections are lost, and the mitochondrial population changes to resemble that present in WAT (Loncar and Afzelius, 1989). These deposits have been termed convertible adipose tissue by Loncar (1989), and can have morphological and functional characteristics of either WAT or BAT depending on the thermoregulatory demands of the animal.

Smaller eutherians, such as rats and mice, also appear to possess this convertible adipose tissue (Loncar *et al.*, 1988a; Loncar 1991b). Only those adipose tissue deposits that expressed the uncoupling protein in the neonatal period appear to be capable of conversion back from white to brown fat in the adult with exposure to certain stimuli. In other fat deposits that are WAT even in the neonate (eg. the epididymal), the uncoupling protein is not expressed (Loncar *et al.*, 1988a,b) although morphologically the cells can change to resemble BAT. In the adult, BAT accumulation has been noted with cold exposure in the cat (Loncar *et al.*, 1986), long term exposure to β-adrenergic stimulation in dogs (Champigny *et al.*, 1991) and the appearance of certain disease states in man (Ricquier *et al.*, 1982; Lean *et al.*, 1986a,b; Soares and Silveira, 1991; Shellock *et al.*, 1985; Ito *et al.*, 1988). The retention of active BAT has also been suggested to be a causative factor of sudden infant death syndrome (Douglas, 1992; Naeye, 1974; Valdes-Dapena *et al.*, 1976).

### 1.3.5 *Species distribution of BAT*

The limits of the species distribution of BAT have still not been fully defined with certainty, although current evidence would suggest that it is restricted to mammals - specifically eutherian mammals. It has been found to be widely distributed in eutherians, from rodents through to primates (Trayhurn, 1989), including those listed in Table 1.3, and is particularly evident and well-developed in hibernating animals, cold-adapted rodents, in overfeeding, and especially in the newborn of a number of mammalian species (Trayhurn and Nicholls, 1986). BAT has been found in 13 of the 21 orders of mammals examined (Rothwell and Stock, 1985) but most

studies have been conducted on small laboratory rodents using histological techniques.

**Table 1.3** Species distribution of BAT based on the immunological identification of uncoupling protein in an adipose tissue (Trayhurn, 1993).

| |
|---|
| Laboratory: |
| mouse - rat - guinea pig - rabbit |
| Hibernators: |
| hamsters (Golden, European, Turkish) - ground squirrels (Richardson's, Columbian, 13-lined) - Pipistrelle bat - edible dormouse |
| Other rodents: |
| Djungarian hamster - wood lemming - wood mouse - Orkney vole |
| Domesticated:(present only in the neonate/young) |
| dogs - sheep - cattle - reindeer (caribou) - red deer - goat |
| Primates: |
| monkeys (Rhesus, Cynomologus, Macaca) - human (newborn and adult) |

The presence or absence of BAT in different species, and its distinguishment from WAT has been undertaken, until recently, using an histological approach. Using this criteria, some investigators have described the presence of BAT in non-eutherian species.

In marsupials and monotremes, controversy as to the presence of BAT has existed for many years. Loudon *et al.* (1985) described the presence of BAT in Bennet's wallabies (*Macropus rufogriseus*) using an histological approach, but Smith and Dawson (1984) claimed that marsupials (*Dasyuroides byrnei*) do not possess BAT. In agreeance with this latter study, Hayward and Lisson (1992) used both morphological and histological techniques to show that all fat deposits in any species from all extant families of marsupials and monotremes (including *Macropus rufogriseus* and *Dasyuroides byrnei*) were typical WAT. Considering this latter evidence, it is probable that marsupials and monotremes do not possess BAT.

In birds, BAT appears to be absent, using both histological (Johnston, 1971; Saarela *et al.*, 1989) and immunological investigative techniques (Saarela *et al.*, 1991). In fish, a heater organ has been found in various species (see Section 1.4.2) but this does not possess either the uncoupling protein of BAT or an analagous

protein and the isolated mitochondria are coupled unlike those from BAT (Block, 1987).

As no other tissue resembling BAT has so far been reported in birds, fish, amphibians or reptiles (Rothwell and Stock, 1985), the uncoupling protein (that necessity for the tissue to be BAT) seems to be specific to the eutherian mammals.

### 1.4 *Contribution of other tissues*

BAT is indisputably a tissue that is capable of high rates of heat production. With the development of transgenic mice, in which BAT has been genetically ablated, its neccessity in homeothermy in eutherian species becomes doubtful. Not only were these mice able to survive the postnatal period which is the greatest period of cold stress, but when raised at normal (24°C) housing temperatures, their body temperature was the same as that of the controls, *i.e.* those that still possessed BAT (Lowell *et al.*, 1993). BAT appears to be an absolute requirement for maintenance of body temperature during acute cold exposure. Lowell *et al.* (1993) demonstrated that although control animals were able to maintain their body temperature when cold stressed (4°C for up to 50 hours), the transgenic mice were not. Their core temperature gradually dropped by 1.7°C over the first 30 hours but remained stable for the next 20 hours of the study.

Although the importance of BAT in mice (a small eutherian) has been shown with regards to its neccessity in the maintenance of homeothermy in response to acute cold stress (Lowell *et al.* 1993), the need for this tissue at normal environmental temperatures seems doubtful. As this tissue also appears to be absent in all but small and neonatal eutherians, it is unlikely that it is the only tissue present that is capable of increased rates of thermogenesis. With all homeotherms (birds, marsupials, monotremes and eutherians) able to maintain a constant body temperature between 35 and 42°C often despite lower environmental temperatures, the presence of a common tissue that is capable of the production of the required heat is suggested.

Tissues that have been proposed to contribute significantly to this required increase in thermogenic rate include vascular tissue and skeletal muscle.

#### 1.4.1 *Vasculature*

When stimulated by various vasoconstrictors, the isolated perfused rat hindlimb exhibits an increase in oxygen consumption in conjunction with an increase in the arterial perfusion pressure (Grubb and Folk, 1976; Richter *et al.* 1982; Colquhoun *et al.*, 1988, 1990; Ye *et al.*, 1990a, 1990b; Hettiarachchi *et al.*, 1992). As both of these induced changes are able to be inhibited by vasodilators such as

nitroprusside, glycerotrinitrate, nifedipine and isoprenaline, it was initially proposed that it was the vasculature that was responsible for the oxygen consumption. Further support for this supposition was gained by the perfusion of the isolated mesenteric arterial bed (Ye *et al.*, 1990a) in which agonist-mediated changes in both perfusion pressure and oxygen uptake are also seen.

These data resulted in the generation of the "hot pipe" hypothesis (reviewed by Colquhoun and Clark, 1991), where it was proposed that marked thermogenesis by the blood vessels occurred both during the constrictive process and in the maintenance of the developed tension. The major assumptions involved in the formulation of this hypothesis were that arterioles have a higher metabolic rate than that described by Paul (1980) for the aorta, and that the 3.4% volume of vascular tissue estimated to be present in the hindlimb is mainly these arterioles.

Further investigation of two parameters - arterial pressure and oxygen consumption - revealed that although they tended to occur together on the addition of a vasoconstrictor, the oxygen effect was dependent on both the dose and the vasoconstrictor that was administered.

Norepinephrine was found to have what has now been referred to as "low" and "high" dose effects. At doses below 1μM, oxygen uptake was stimulated, but at doses above this, the oxygen uptake was inhibited back to and below the original basal level. Serotonin, another vasoconstrictor, only created an inhibition of the basal oxygen uptake, despite accompanying increases in perfusion pressure still occurring (Dora *et al.*, 1991). Further work by Dora and colleagues (1992) showed that high dose norepinephrine- or serotonin-induced vasoconstriction was not significantly affected by either hypoxia or the addition of cyanide or azide, whereas that of low dose norepinephrine was. This data suggests that the vasculature, and the development and maintenance of the induced pressure response, is not solely responsible for the increased oxygen consumption.

The ability of smooth muscle to respond to high doses of epinephrine and norepinephrine under hypoxic and anoxic conditions has been previously observed in earlier studies on larger arterial segments (Altura and Altura, 1976; DeMey and Vanhoutte, 1983; Shibata and Briggs, 1967). This anaerobic capability of smooth muscle for the development and maintenance of a vasoconstriction equal to or greater than that seen under aerobic conditions undermines the "hot pipes" hypothesis as according to it, any increase in the perfusion pressure should be accompanied by an increased oxygen consumption due to the work needed to obtain and maintain the vasoconstriction.

As vasoconstriction and oxygen consumption now appear to be unrelated, the data suggests that the vasculature is responsible for substrate delivery. This alternate

explanation implies that vasoconstrictors are actually regulating the pattern of blood (or perfusate) flow to the muscle fibres, and it is these which are responsible for the increased oxygen consumption. Using this reasoning, selected vasoconstrictors at certain concentrations would improve the flow through "nutritive" capillaries in the muscle bed, whereas a different vasoconstrictor or the same vasoconstrictor at a different concentration could severely inhibit this flow (see Figure 3.3).

The presence of high-flow (or non-nutritive) capillaries in contracting muscle in rabbits and dogs (Harrison *et al.*, 1990) and their effect on local oxygen supply and blood flow regulation suggests that although they account for only 13% of the total capillary density (Potter and Groom, 1983) they could be capable of carrying 70% of the flow under resting conditions. The diameter of these non-nutritive capillaries would not differ greatly from that of the remaining "nutritive" capillaries. These diameters have been estimated to be 4-6μm and 7-8μm for nutritive and non-nutritive capillaries respectively (postulated by Harrison *et al.*, 1990 from the data of Potter and Groom, 1983). Changes in the proportion of the total blood (or perfusate) flow through these two capillary beds could then be affected by either changes in circulating vasoconstrictor and vasodilator concentrations, or changes in sympathetic nervous system activity.

### 1.4.2 *Skeletal muscle*

In various species of fish, a thermogenic tissue that possesses both a rich blood supply and numerous mitochondria has been described (Carey, 1982). These fish, (swordfish, sailfish, white marlin, blue marlin, blue-fin tuna, and striped marlin) maintain brain temperatures of up to 10°C above that of their environment and the rest of the body which remains at water temperature (Carey, 1982; Block, 1986, 1987). This tissue is located beneath the brain and adjacent to the eyes. Using both morphological and biochemical techniques, Block (1986) showed that the heater organ and muscle were closely related and that it was in fact derived from the superior rectus eye muscle. During modification from muscle to heater organ, the most conspicuous changes are the loss of most of the contractile components of the normal muscle cell and hypertrophy of the internal membranes that regulate calcium ion movements (Block, 1994). Together with functional changes in the eye muscles, both the muscle tissue structure and blood vasculature are altered to allow the heat-generating cells to maintain a large surface area in contact with the blood supply (Block, 1986). The heat producing portion of the eye muscle is supplied with blood through a counter-current heat exchanger that originates from the carotid artery. In this organ, it seems that heat is produced via stimulation of mitochondrial oxidation

rates by a cytoplasmically based mechanism that could be a $Ca^{2+}$ ATPase (Block, 1994 and references therein).

The evolution of this heating organ, in essence the development of cranial endothermy, in these fish species has been linked with selection for thermal niche expansion (Block *et al.*, 1993). Despite the similarities in function between BAT and this heater organ, the biochemical pathways involved in the generation of heat appear different, in part due to their evolutionary derivation from different tissues - adipose and muscle (Block, 1987). As this tissue is unlikely to be BAT and although derived from it is not now muscle, it does suggest the possibility of a role for skeletal muscle in whole body thermogenesis.

Skeletal muscle, which in rodents and humans comprises approximately 40% of the total body mass (Foster and Frydman, 1978; Dickerson and Widdowson, 1960), has also been proposed as a tissue capable of contributing significantly to whole body thermogenesis. When working skeletal muscle has the potential to be markedly thermogenic, due in part to its sheer mass.

During long term exposure to cold, the mechanism for heat production switches from shivering to non-shivering thermogenesis (Himms-Hagen, 1976; Jansky, 1973). When exposed to cold (5°C), an increase in both shivering and metabolic rate is noticeable in rats raised at both thermoneutrality (23-25°C) and 5°C. Although both groups display these symptoms, those raised at 5°C have a higher metabolic rate and body temperature than those raised at thermoneutrality and they shiver less (Sellers *et* al., 1954; Hart *et al.*, 1956; Gautier *et al.*, 1991). Norepinephrine has been found to play a vital role in this increased non-shivering thermogenesis during and after the period of cold acclimation.

Norepinephrine turnover or sympathetic drive increases (15-50%) to skeletal muscle with long-term cold exposure (Dulloo *et al.*, 1988). This increase in sympathetic drive has an effect on the oxygen consumption of the hindlimb. The increased sympathetic drive has two effects- to increase the blood flow to the tissue and to stimulate tissue oxygen consumption.

On acclimation to cold, skeletal muscle capillarity has been found to increase. In the rat, Heroux and St. Pierre (1957) found that vascularization in the heart, liver and white fibre type muscles was unaffected by 4 weeks of cold exposure. In the red fibre type muscles, soleus and regions of the gastrocnemius, there was an absolute increase in the number of capillaries per $mm^2$, with densely vascularized subregions of these muscles becoming approximately 50% larger after cold acclimation. Heroux and St. Pierre (1957) interpreted this as an indication of a higher metabolism, and possibly an increased chemical thermogenesis in the red fibre type muscles. In the guinea pigs after 18 weeks cold exposure, Sillau *et al.* (1980) found this same

increased capillarity in the soleus and gastrocnemius muscles and also concluded that this suggested an improved capacity for oxygenation, a response that correlates well with the increased oxygen consumption and thermogenesis seen during prolonged cold exposure. A 46% increased cardiac output has also been noted with cold exposure (Jansky and Hart, 1968) and blood flow to the muscular organs increased significantly (heart and diaphragm by 126% and skeletal muscle by 87%).

Adaptive changes in skeletal muscle mitochondria with cold-acclimation have been well documented. The mitochondria become smaller and more numerous, but they still show normal ADP/O ratios and are not uncoupled (Himms-Hagen *et al.*, 1975, 1976). Mitochondrial protein synthesis is altered with cold-acclimation, with reductions in the half-lives of some mitochondrial proteins in skeletal muscle (Bukowiecki *et al.*, 1971, 1976). Increases in the oxidative metabolism of the mitochondria occur with cold acclimation. All the mitochondrial cytochromes, succinate dehydrogenase activity, cytochrome oxidase activity and the respiration rate have been shown to increase with cold-acclimation in the guinea pig (Kinnula *et al.*, 1983).

The importance of the vasculature to resting skeletal muscle thermogenesis is well known. Vasoconstrictor-mediated effects on metabolism are not seen in isolated incubated muscle preparations (Dubois-Ferriere and Chinet, 1981; Hettiarachchi *et al.*, 1992). When delivery of the vasoconstrictors is via the vascular route, however, they are able to induce a marked change in the rate at which oxygen is used. In the perfused rat hindlimb at 37°C, the norepinephrine-induced effects are marked, with rapid increases from a basal oxygen consumption of $24\mu mol.g^{-1}.h^{-1}$ to a maximum of $35\mu mol.g^{-1}.h^{-1}$ (S. Rattigan personal communication, 1994). In the rat, calculations from these figures indicate that skeletal muscle could contribute about 36% of the total oxygen uptake that could be contributed by BAT.

An increase in sympathetic drive can be mimicked by the infusion of norepinephrine into the blood or perfusate. *In vivo*, Foster and Frydman (1979) have shown this to create a 30% increase in blood flow and a 60% increase in muscle oxygen consumption. In contrast to these observed changes, Jansky and Hart (1963) showed that with no increase in hindlimb blood flow *in vivo*, norepinephrine infusion or cold exposure caused a doubling of the skeletal muscle oxygen consumption. In that study, the stimulation of thermogenesis due to norepinephrine infusion resulted in an increase in whole body, but not leg, temperature, and despite similar increases in oxygen consumption due to cold exposure, the rats' body temperature was seen to decrease slightly even though leg temperature remained constant. When norepinephrine has been infused into the rat hindlimb perfused at a constant flow rate

this same response (i.e. an increase in oxygen consumption) has been noted (Section 1.4.2).

These data suggest that the increase in skeletal muscle metabolism is due to an increase in the amount of oxygen extracted from the blood, with the possibility that the increased extraction is somehow stimulated by norepinephrine. Similar responses to the infusion of low dose norepinephrine have been found *in vivo*. Boorstein *et al.* (1994) found that norepinephrine caused increases in both the delivery and usage of oxygen in dogs.

Other researchers have found difference *in vitro* between the responses of skeletal muscle from thermoneutral and cold-acclimated rats. Although the basal oxygen uptake ($3.2\mu mol.min^{-1}.leg^{-1}$) was found to be unaffected by cold exposure (4° C for up to 25 days), Shiota and Masumi (1988) found that the norepinephrine-stimulated change in oxygen consumption was increased when the rat hindlimb was perfused at 32°C (from approximately 3.4 to 4.9 $\mu mol.min^{-1}.leg^{-1}$).

Similar responses to vasoconstrictors have been recently found in a marsupial (Ye *et al.*, 1995). The response to norepinephrine was found to be dependent on the constriction of the vasculature as it was inhibited by both an $\alpha$-antagonist and general vasodilators, but unaffected by $\beta$-antagonists. As marsupials do not appear to possess BAT, this response is a further indication of the importance of skeletal muscle in the thermoregulatory ability of homeotherms.

## 1.5 *Aims*

With the demonstration of the importance of the vascular system in the thermogenic contribution of skeletal muscle and the ability of the perfused skeletal muscle system to increase its oxygen consumption on the infusion of certain vasoconstrictors (Clark *et al.*, 1995), two different but related questions were raised.

Does BAT possess a vascular component that contributes directly to, or controls, thermogenesis? Are there elements of BAT thermogenesis in muscle?

The first aim of this study was to develop and examine a perfused BAT preparation for the presence of an α-mediated thermogenic component similar to that already demonstrated in the perfused hindlimb (Richter *et al.*, 1982).

The other aim of this study was thus to investigate the possibility of the presence of a physiological uncoupler or recoupler in skeletal muscle, that is possibly produced or removed by vasconstriction and/or redistribution of perfusate flow through the muscles. This was done by examining the differences between liver and skeletal muscle mitochondria and variations in their responses to different parameters that could be responsible for uncoupling similar to that seen in BAT.

# Chapter 2

## Is the Vasculature involved in the control of BAT thermogenesis?

### 2.1 *Introduction*

A major problem for biochemical studies of BAT has been that a satisfactory *in vitro* preparation has not been available for studies of thermogenesis of the whole tissue (Cooney and Newsholme, 1984). BAT thermogenesis has been investigated *in vitro* using isolated adipocytes and BAT slices (both perifused and incubated), but these methods allow the examination of only part of the action of the whole tissue. Any vascular involvement in the tissues' thermogenic response is ignored in all of these *in vitro* methods.

Measurement of the thermogenic capacity of BAT has been hampered by its presence in small, scattered deposits (Himms-Hagen, 1989; Foster *et al.*, 1980). The BAT depots that have been most extensively examined are the interscapular and axillary due to their greater size, superficial location, and hence accessibility. Despite these advantages, interscapular BAT possesses multiple afferent and efferent blood vessels and, although it has been perfused (Seydoux *et al.*, 1975), the direct cannulation, isolation and specific perfusion of BAT in this region has not been achieved.

Other deposits, the periaortic and perirenal, have been shown to be important sites of thermogenesis both at low doses of norepinephrine (NE) and in unanaesthetized rats subjected to mild cold (Foster, 1986). Examination of alternate deposits in the rat revealed an opportunity for the selective perfusion of the periaortic BAT deposit.

Studies using the perfused rat hindlimb have found that norepinephrine increased oxygen uptake always in association with vasoconstriction and that both effects were blocked by the nitrovasodilator, sodium nitroprusside (Colquhoun *et al.*, 1988). Further, the stimulatory effect on oxygen uptake was evident with other agonists that also caused vasoconstriction such as vasopressin and angiotensin II (Colquhoun *et al.*, 1988). In the perfused rat hindlimb micromolar doses of isoproterenol partially dilated and did not increase oxygen uptake on its own, but completely inhibited the increase in oxygen uptake and perfusion pressure by either angiotensin II or norepinephrine (Colquhoun *et al.*, 1990). Furthermore, an increase in perfusate flow rate also increased oxygen uptake by the hindlimb and the stimulatory effect of norepinephrine on oxygen uptake and pressure development occurred at all flow rates

(Ye *et al.*, 1990b). To account for these observations in the hindlimb, it has been proposed that the increase in oxygen uptake in association with vasoconstriction results from either work done by the vascular tissue in constricting and holding pressure or from the vascular system, under the influence of specific vasoconstrictors, directing oxygen carrying medium to specific oxygen consuming regions in skeletal muscle. This vasoconstrictor-mediated increase in oxygen uptake has also been shown to occurr in constant-flow perfused kidney, intestine and mesenteric artery arcade (Ye *et al.*, 1990a).

In the present study, a technique for the perfusion of periaortic BAT has been developed with a view to assessing the involvement of the vascular system in the tissues' thermogenesis, either as a contributor (e.g. as working vascular tissue; Colquhoun *et al.*, 1988; Ye *et al.*, 1990a,b; Colquhoun and Clark, 1991), or as controlling influence (via nutrient access).

## 2.2 *Materials and Methods*

### 2.2.1 *Perfusion system*

Male hooded Wistar rats (220 - 270 g body wt) were housed in groups of 3-4 at 20-22°C with a light:dark cycle of 12h:12h. They were fed a commercial diet (21.4% protein, 4.6% lipid, 68% carbohydrate, 6% crude fibre, with added vitamins and minerals; Gibsons, Hobart) with free access to water. In early experiments rats were also allowed access for 2 weeks to a solution of sucrose (37%, w/v). This increased the mass of BAT in the periaortic region by approx. 40%, but to a lesser extent than it did for the interscapular deposit, which doubled. The sucrose drinking option stimulus was deemed necessary initially because of the small size of BAT in the periaortic region. However, as surgical techniques improved, this small mass was later found to be of an adequate size for perfusion and measurement of oxygen uptake and so the sucrose drinking option was discontinued. Basal and stimulated $\dot{V}O_2$ from perfused periaortic BAT from rats with or without the sucrose drinking option showed no significant differences when calculated per gram of tissue and so the data was pooled.

Animals were anaesthetized with sodium pentobarbitone (6 mg/100 mg body wt) combined with heparin (500 Units) administered intraperitoneally. The thorax was opened, the heart and lungs removed, and the aorta exposed. A teflon cannula (16G, 1.70 mm O.D. and 1.30 mm I.D.) was inserted into the transected aortic arch, in the direction of blood flow, past the point of bifurcation of the left subclavian artery and ligated (Fig. 2.1 (**1**)). The aorta was then ligated proximal to the diaphragm (**2**), and

perfusion commenced at 1.0±0.1 ml.min$^{-1}$. The intercostal vessels were sequentially ligated (starting at **3**) in the caudal direction, 3 to 5 mm laterally away from the aorta, and an incision was made in the Azygous vein (**4**) to allow free venous drainage. The preparation was then carefully removed from the thoracic cavity, and placed into a buffer-filled vessel (5.3 cm x 0.9 cm diameter) which was inverted after connection to the perfusion system (Fig. 2.2).

The perfusate consisted of a Krebs-Ringer bicarbonate buffer, composition (mM): NaCl 119, KCl 4.8, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 8.3, EDTA 0.1, $CaCl_2$ 1.27 and 1% (w/v) bovine serum albumin. Albumin was used after dialysis against water five times, after charcoal treatment (Chen, 1967) or as received (fatty acid-free) with no further treatment. Perfusate was pumped from a reservoir kept at 4°C through a silastic tube oxygenator continuously equilibrated against a gas mixture of 95% $O_2$ and 5% $CO_2$. The temperature of the perfusate was brought to 37°C by passage through a heat exchange coil. The perfusate flow rate was maintained constant at 1 ml.min$^{-1}$ or at the values indicated. Perfusion pressure was measured in the pump outflow line proximal to the preparation. As all perfusions were conducted at a constant flow rate, changes in perfusion pressure were assumed to reflect changes in net vascular resistance. The venous effluent flowed through a 0.5 ml thermostatically controlled chamber (37°C) containing a Clark-type oxygen electrode. All perfusion tubing other than the cannula was polyethylene.

Solutions of the agonists, antagonists, or vehicle (0.1% ascorbic acid in isotonic saline) were freshly prepared from the dry powders and were infused at 10 µl.min$^{-1}$, into the perfusion line proximal to the arterial cannula.

### 2.2.2 *Determination of the extent of perfusion of periaortic BAT*

At the conclusion of each experiment, particulate Indian ink was injected through the infusion port under basal pressure and flow conditions. Perfused and non-perfused regions were excised, blotted dry and weighed. For some preparations visualization of the perfused areas was made microscopically in fresh-frozen 10 µm sections cut in a Slee type R freezing microtome. On occasion, the ink was infused simultaneously with either norepinephrine or isoproterenol.

### 2.2.3 *Lactate dehydrogenase leakage*

Perfusate samples collected during the course of a perfusion, as well as tissue samples (blotted dry) taken both before and after perfusion, were stored at -80°C prior to analysis for lactate dehydrogenase. The protocol followed was essentially that of Bergmeyer (1983). Briefly, tissue samples (20 mg) were homogenized in 2 ml phosphate buffer (0.1 M pH 7.0) for approximately 30 seconds using an Ultra Turrax

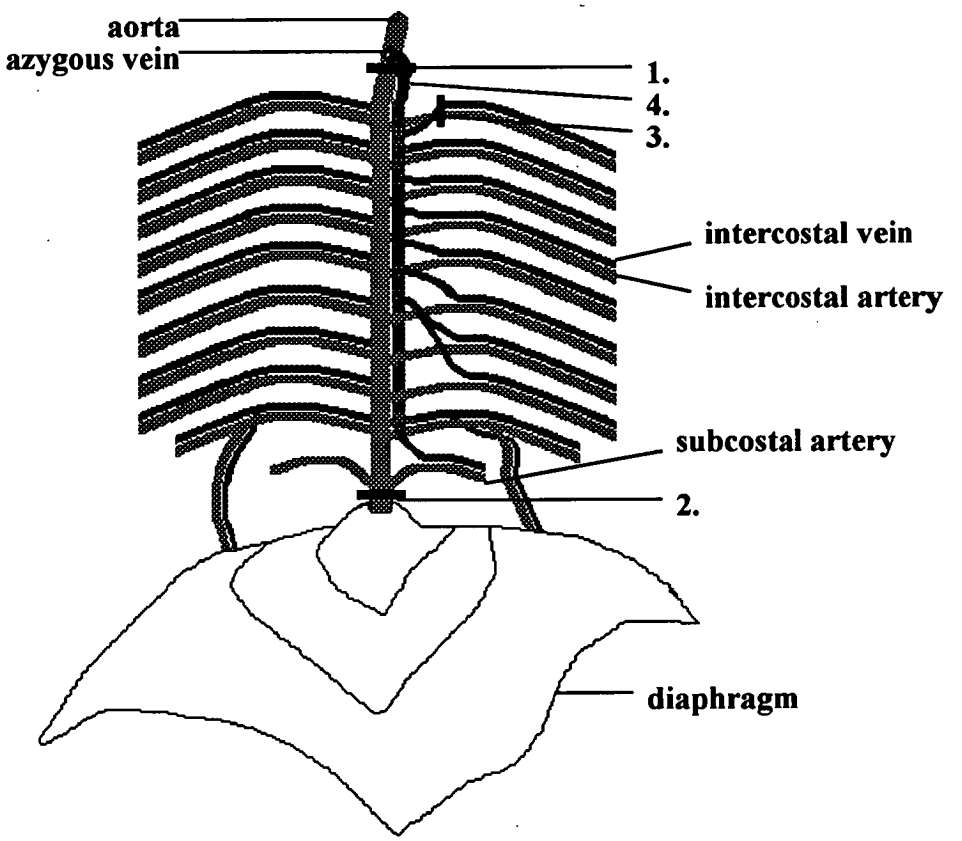


**Figure 2.1** Vessels ligated during the surgical preparation of the isolated periaortic BAT (modified from Greene, 1968). A teflon cannula was inserted into the aorta, past the point of bifurcation of the left subclavian artery, and ligated (1). The aorta was then ligated proximal to the diaphragm (2) before the intercostal vessels were sequentially ligated (starting at 3) in the caudal direction, 3 to 5 mm laterally away from the aorta. An incision was made in the Azygous vein (4) to allow free venous drainage of the perfusate.

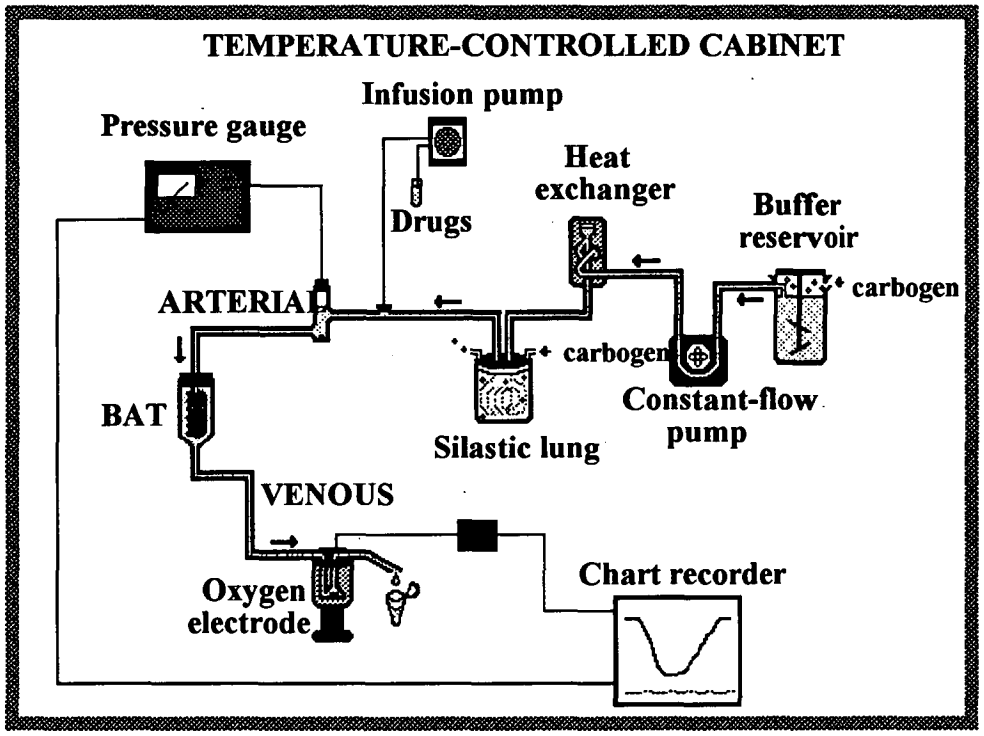


**Figure 2.2** Periaortic BAT perfusion apparatus.
Carbogen-gassed buffer was pumped at a constant flow rate by a peristaltic pump from the reservoir through the apparatus. The media was brought to 37°C and fully gassed by passage through a water-jacketed glass heat exchange coil and a carbogen-gassed silastic tubing oxygenator. Perfusion pressure was continuously monitored in a sidearm mixing chamber/bubble trap proximal to the aorta. The oxygen content of the venous effluent was measured continuously using an in-line, water-jacketed Clark-type oxygen electrode. Carbogen, 95% $O_2$ - 5% $CO_2$.

homogenizer. Aliquots (100 µl of perfusate and 10 µl of tissue) were then added to cuvettes containing phosphate buffer, 0.25 mg pyruvate and 0.5 mg NADH, and the enzyme activity followed at 339 nm using a Cary spectrophotometer.

### 2.2.4 *Tissue high energy phosphate content*

Samples of interscapular BAT were excised rapidly with little or no ischemia, freeze-clamped in liquid nitrogen-cooled tongs and stored at -80°C. An unavoidable delay of approximately 75 s ischemia occurred during the removal of the periaortic BAT. Periaortic BAT that had been perfused for 30 min as well as interscapular BAT that had been left undisturbed in a dead animal for 30 min (i.e. 30 min of ischemia) were also freeze-clamped.

Tissue samples were homogenized in 0.24 M perchloric acid (30 mg tissue.ml$^{-1}$), centrifuged (2,500x *g*) and a portion of the supernatant neutralized with 1 M-$K_2HPO_4$ (pH 6.0). After recentrifuging, an aliquot (20 µl) was used for resolution by HPLC using an isocratic technique modified from Sellevold *et al.* (1986). The HPLC system consisted of a Waters chromatography pump, Waters Model 440 and 441 detectors and a Waters Rad-Pak C-18 column. Creatine compounds were detected at 214 nm and adenine nucleotides at 254 nm. The mobile phase consisted of 0.22 M $KH_2PO_4$, 3 mM tetrabutyl ammonium phosphate and 3.5% acetonitrile, adjusted to pH 6.25 with 1 M KOH. Flow rate was 1.5 ml.min$^{-1}$.

### 2.2.5 *Vascular casting*

Casting of perfused periaortic BAT was achieved by halting perfusate flow and rapidly injecting the casting mix via a 3-way tap, with the infusion pressure being sufficient to force the material through the vasculature but not into the extra vascular spaces. Vertex denture material, self-curing, Type II, Class I, nominal bead diameter 30 µm (1.6 ml liquid and 1 ml powder) from Dentimex, Holland, was infused until no further flow could be obtained. The vascular cast produced was then allowed to set for 1-2 h before digestion in 40% (w/v) KOH at 60°C for 2-3 days to remove the surrounding tissue.

### 2.2.6 *Electron microscopy*

BAT from either the periaortic or interscapular region was removed from anaesthetized rats, cleaned of extraneous white adipose tissue and muscle and cut into cubes (<1 mm$^3$). Samples were fixed for 2 h in 3% glutaraldehyde in 0.08 M sodium cacodylate buffer at pH 7.2, and 4°C, washed in buffer, and post-fixed in 1% (w/v) $OsO_4$ in distilled water for 1 h at room temperature. The cubes were then washed in

water, post-fixed in 4% uranyl acetate in water for 1 h and dehydrated in a graded series of alcohol. The tissue was then embedded in Epon.

Thin sections were cut with a diamond knife on an LKB Nova ultramicrotome and transferred to Pormar coated slot grids, stained with lead citrate and aqueous uranyl acetate, and observed with an Hitachi H300 transmission electron microscope operated at 70 kV.

Morphometric assessment was made from EM photographs using at least 20 randomly selected pictures of either periaortic or interscapular BAT tissue.

### 2.2.7 *Enzyme activities of BAT*

BAT was removed, cleaned of extraneous tissue, homogenised in the appropriate buffer using a Potter-Elvehjem homogenizer and assayed at 37°C using a Cary spectrophotometer for hexokinase (HK; Bergmeyer, 1983), phosphofructokinase (PFK; Layzer, 1975) and cytochrome oxidase (COX; Wharton and Tzagoloff, 1967).

Briefly, for HK analysis, a Triethanolamine (50 mM, pH 7.6) buffer was used, with 8 mM glucose, 100 mM $MgCl_2$, 2 mg NADP+, 10 μg glucose-6-phosphate dehydrogenase and 30 mM ATP added for measuring the activity at 340 nm. For PFK analysis, a buffer containing 100 mM Tris-HCl (pH 8.0), 4 mM $MgSO_4$, 5 mM $(NH_4)_2SO_4$ and 2 mM dithiothreitol was used, with 30 mM fructose-6-phosphate, 0.5 mg NADH, 1.8 units.ml$^{-1}$ aldolase, 1.8 units.ml$^{-1}$ triosephosphate isomerase, 0.9 units.ml$^{-1}$ a-glycerophosphate dehydrogenase and 30 mM ATP added for measuring the activity at 340 nm. COX activity was analysed at 550 nm after homogenization in a phosphate buffer (50 mM), in 5 mM phosphate buffer with 0.05% reduced cytochrome c.

### 2.2.8 *Oxygen uptake calculations*

The rates of oxygen uptake ($\dot{V}O_2$) were calculated by multiplying the arteriovenous difference (A-V) in oxygen content by the flow rate of the perfusate per gram of periaortic BAT. $\dot{V}O_2$ in μmoles.h$^{-1}$.g$^{-1}$ was calculated as follows:

$$\underset{(\mu moles.h^{-1}.g^{-1})}{\dot{V}O_2} = \frac{(A\text{-}V) \times 1.256^{*} \times 60 \times \text{flow rate } (ml.min^{-1})}{\text{BAT mass}^{\dagger} \times 22.4^{\#}}$$

* 1.256 (mmol.l$^{-1}$.mmHg$^{-1}$) is the Bunsen coefficient for plasma at 37°C (Christoforides *et al.* 1969).

† Measured as the blotted, dye coloured proportion of the present BAT mass at the conclusion of the experiment (see Section 2.2.2).

\# At standard temperature and pressure the volume of 1μmol of oxygen is 22.4μl.

### 2.2.9 *Agonists and antagonists*

Norepinephrine-HCl, isoproterenol-HCl, DL-propranolol-HCl and phentolamine were from Sigma. BRL 35135 was a generous gift from Dr. M.A. Cawthorne, SmithKline Beecham, Epsom, U.K. Sodium nitroprusside was a generous gift from Merck, USA.

### 2.2.10 *Statistics*

Values are reported as means ± SEM and are expressed in terms of g wet weight of perfused tissue unless indicated otherwise. Curves were fitted using the Sigma Plot program (Version 4.0, Jandel Scientific). Statistical significance of difference between groups of data was assessed using the unpaired 2-tailed Student's *t*-test.

## 2.3 *Results*

### 2.3.1 *Characterization of perfused periaortic BAT*

In these experiments periaortic BAT from the rat was perfused and its properties assessed. A typical preparation was approximately 15 mm long, 3 mm wide and comprised 160 mg (72 mg perfused and 66 mg unperfused) of BAT and 22 mg aorta. Fig. 2.3A shows the morphology of the preparation as illustrated from a dental acrylate cast. The principal component is the aorta with intercostal arteries and the arterial vessels (X, Y and Z) which supply the periaortic tissue. These latter vessels are depicted in both left and right aspects of the cast (Fig. 2.3A) and run parallel to the aorta. Some of the intercostal arteries have filled with casting material and can be seen emerging from, and running at right angles to, the aorta. It is important to note that only some of the arterial vessels are shown in a cast of this kind. The particle size of dental acrylate is approximately 30 μm and thus passage through capillaries to the venous vessels does not occur.

Indian ink was used routinely at the end of each perfusion to determine the region perfused; this represented 44±2% (n=54) of the total mass of the preparation (Table 2.1). A low power section (Fig. 2.3B) shows the extensive Indian ink-filled vasculature of a successfully perfused ventral region of periaortic BAT; non-perfused regions were largely restricted to areas close to the ligatures that secured the cannula and tied off the intercostal vessels.

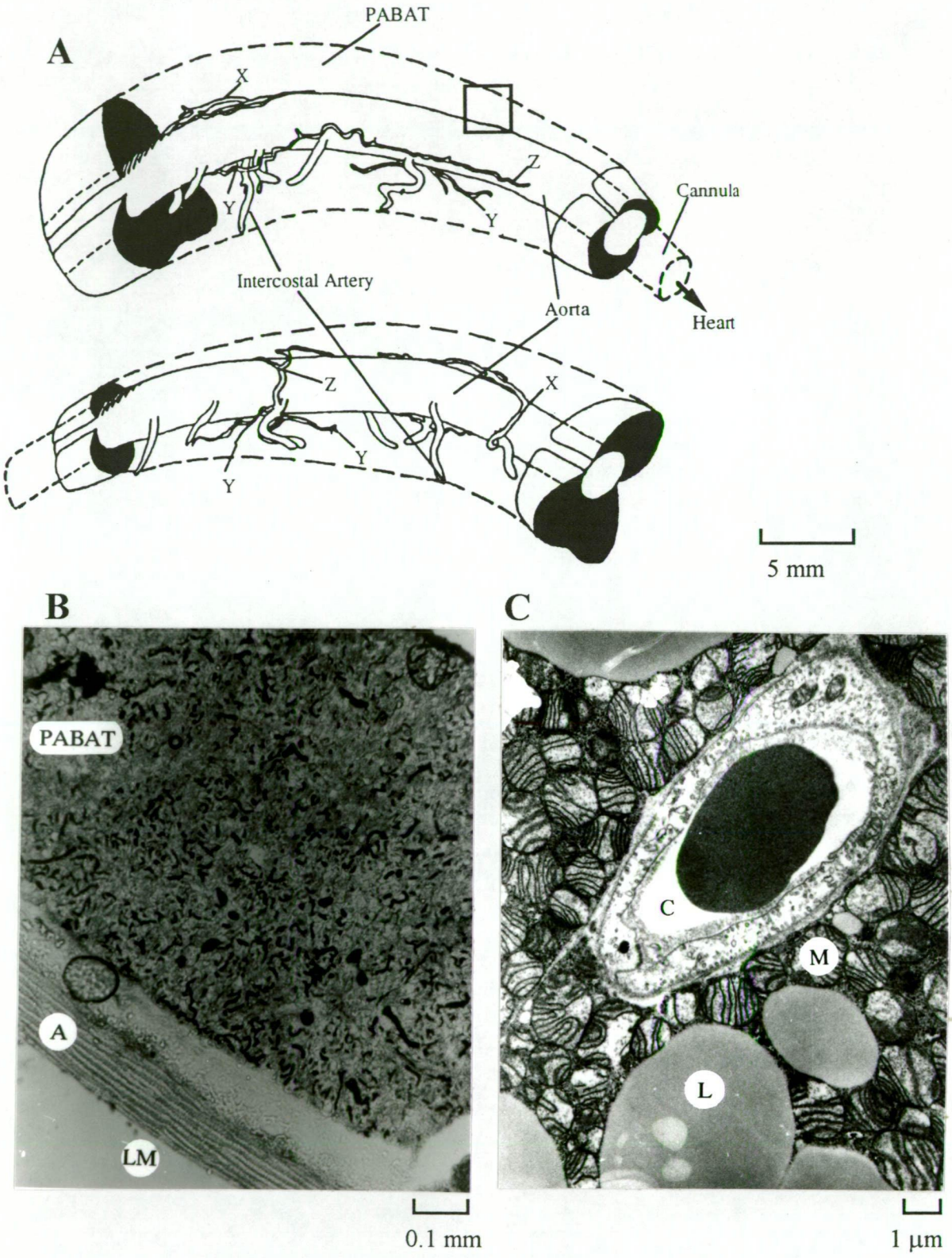


**Figure 2.3** Morphology of periaortic BAT of the rat. A, Schematic illustration (lateral views) of a dental acrylate cast of the periaortic BAT showing some of the arterial vessels (X, Y and Z; ≥30 μm) for both the left and right aspects of the same cast. B, inset of A, showing a longitudinal section of Indian ink infused ventral periaortic BAT, with the lumen (LM), aorta (A) and periaortic BAT. Indian ink was infused at the end of the perfusion to check the area perfused. C, transmission electron micrograph of periaortic BAT showing dense mitochondria (M) and lipid droplets (L) surrounding a capillary (C).

**Table 2.1** Composition of perfused periaortic BAT preparations

| | Mass (mg) | % of Total Mass |
|---|---|---|
| Brown adipose tissue | | |
| Perfused* | 72.2 ± 6.8 | 44 ± 2 |
| Unperfused† | 65.8 ± 5.1 | 41 ± 3 |
| Aorta | 22.3 ± 1.5 | 15 ± 1 |

NOTES: * The perfused region was assessed by infusion of Indian ink. †Most of the unperfused tissue was either around the ligatures on the intercostal vessels or occluded by the cannula ligature. Data are mean ± SEM for n=54 preparations from rats weighing 253±2 g.

Viability of the preparation was assessed by determining lactate dehydrogenase leakage during perfusion as well as tissue concentrations of high energy phosphate compounds before and after perfusion. In general lactate dehydrogenase leakage did not exceed 2%/h of the total present in an unperfused sample (data not shown). Table 2.2 shows that the energy charge of periaortic BAT after 30 min perfusion closely resembled freeze-clamped interscapular BAT *in vivo*. A decrease in ATP and a build-up in AMP occurred rapidly when BAT was subject to ischemia. The values obtained in the present study are similar to those of Ma and Foster (1984), where the energy charge was 0.78 and the total adenine nucleotides were 3.89 μmol.g dry wt.$^{-1}$ (calculated using the wet/dry weight ratio for interscapular BAT given in Table 2.2). These values (Table 2.2) are probably unphysiologically low. To obtain expected *in vivo* values would probably necessitate the freezing of the tissue *in situ* without prior manipulation (for example removal of the overlying WAT in the interscapular region). Even during the unavoidable delay in removing the periaortic BAT (approx. 75 s) the energy charge decreased to 0.53. There was no significant loss in total adenine nucleotides from periaortic BAT even after 30 min perfusion (Table 2.2), in keeping with only 1% leakage of lactate dehydrogenase over the same period (data not shown). Table 2.2 also shows that edema did not occur and the wet/dry wt ratio after removal of surface fluid was similar to freshly sampled tissue.

Comparisons of periaortic and interscapular BAT were made. Examination of periaortic BAT at the electron microscope level (Fig. 2.3C) showed abundant mitochondria and lipid droplets within cells and numerous capillaries amongst the cells. Morphometric assessment of EM sections from periaortic and interscapular BAT showed no significant difference between the two deposits with regard to cell diameter, lipid content, mitochondrial size, or number of cristae per mitochondria

**Table 2.2** High energy phosphate concentrations in samples of BAT

| Tissue origin | n | Wet/dry wt. | Metabolite concentration (μmol/g dry wt.) ATP | ADP | AMP | Σ Ad | Energy charge |
|---|---|---|---|---|---|---|---|
| PABAT *in vivo** | 6 | 2.10±0.12 | 1.15±0.14 | 1.68±0.09 | 0.92±0.13 | 3.75±0.20 | 0.53±0.03 |
| PABAT - basal perfusion (30 min) | 4 | 4.80±0.20† | 2.14±0.24 | 1.31±0.11 | 0.44±0.17 | 3.89±0.13 | 0.72±0.05 |
| IBAT *in vivo* | 7 | 2.59±0.19 | 2.02±0.27 | 1.40±0.06 | 0.39±0.05 | 3.81±0.23 | 0.71±0.03 |
| IBAT ischemia (30 min) | 6 | 2.59±0.19 | 0.29±0.08 | 0.72±0.16 | 1.85±0.67 | 2.85±0.55 | 0.27±0.06 |

*Although indicated to be *in vivo* excision of this material entails approx. 75 s ischemia compared with little or no ischemia for the removal of IBAT.

†To avoid delay tissue was not cut open and blotted dry before freeze clamping; for samples that were blotted the wet/dry wt was 2.41±0.13 (n=3).

Σ Ad is the adenine nucleotide sum.

**TABLE 2.3** Properties of periaortic and interscapular BAT of the rat.

| | Periaortic | Interscapular |
|---|---|---|
| Enzyme activity ($\mu mol.min^{-1}.g^{-1}$) | | |
| Hexokinase | 14.3±3.1 (6) | 10.3±1.5 (6) |
| Phosphofructokinase | 7.0±4.9 (6) | 7.0±2.3 (6) |
| Cytochrome oxidase | 162±25 (3) | 135±26 (3) |
| Mitochondrial diameter (μm) | 0.78±0.06 (3) | 0.77±0.09 (3) |
| No. cristae/mitochondrial section | 6.24±0.60 (3) | 6.84±0.92 (3) |
| Lipid droplet diameter (μm) | 5.08±0.25 (3) | 6.70±0.85 (3) |
| Cell diameter (μm) | 22±1 (3) | 24±2 (3) |
| % mitochondrial content | 29±1 (3) | 24±2 (3)* |
| % lipid content | 57±3 (3) | 61±6 (3) |

NOTE: Tissue samples were taken from the same rats and data are mean ± SEM with the number of preparations assayed given in parentheses. *, $P<0.05$.

(Table 2.3). Mitochondrial content was significantly higher in the periaortic BAT which would indicate the potential for greater thermogenesis than BAT found in the interscapular region. Enzyme activities for hexokinase and cytochrome oxidase tended to be higher in periaortic BAT, but this was not significant (Table 2.3).

### 2.3.2 $\dot{V}O_2$ *and the effects of norepinephrine and isoproterenol*

After reaching steady state (20 - 30 minutes), $\dot{V}O_2$ was constant throughout the perfusion at 64.3±7.4 $\mu mol.h^{-1}.g$ wet $wt^{-1}$ (n=53) unless perturbed by agonist addition. Arterial $PO_2$ was 571±8 mm Hg (n=53); basal venous $PO_2$ was 524±10 mm Hg (n=53) and this decreased to 450±14 mm Hg (n=53) with maximal stimulation. The use of different albumins (dialyzed, charcoal treated or fatty acid-free) did not affect the basal or stimulated $\dot{V}O_2$. Basal perfusion pressure was also constant throughout each perfusion at 65±3 mm Hg (n=53) but, for unknown reasons, varied between preparations (range = 37 to 119 mm Hg). If the starting perfusion pressure exceeded 120 mmHg, the preparation was rejected. This pressure was chosen as it is the rats' *in vivo* arterial blood pressure, and any basal perfusion pressure above this may have adversly affected the perfused tissue.

Fig. 2.4 shows representative time courses for the effect of 50 nM norepinephrine and 100 nM isoproterenol on $\dot{V}O_2$ by the isolated constant-flow (1.0 $ml.min^{-1}$) perfused periaortic BAT. Responses were rapid and, allowing for flow delay and oxygen electrode response time, were apparent within 30 s after agonist addition. The increase in $\dot{V}O_2$ due to each agonist was slower in reversal than onset and the stimulatory effect of isoproterenol persisted for at least 20 min after removal (Fig. 2.4). Perfusion pressure changes for norepinephrine were variable with no consistent trend. For a total of 43 perfusions, 16 showed a reversible increase, 19 a reversible decrease and 8 showed no change in pressure as a result of norepinephrine addition. Regardless of direction, the pressure change did not exceed 33 mm Hg. Isoproterenol had no significant effect on perfusion pressure.

Fig. 2.5 shows dose-response curves for norepinephrine and isoproterenol-mediated increases in $\dot{V}O_2$ by perfused periaortic BAT. Half-maximal effects occurred at 12 nM norepinephrine and 8 nM isoproterenol. Responsiveness was similar for each agonist, giving rise to a maximal $\dot{V}O_2$ of approx. 150 $\mu mol.h^{-1}.g$ wet $wt^{-1}$. A maximal dose of the $\beta_3$ agonist, BRL 35135 (45 nM) increased $\dot{V}O_2$ by 107.2 ±20.6 $\mu mol.h^{-1}.g^{-1}$ (n=11), an increase similar to that produced by a maximal dose of norepinephrine.

In the data shown in Fig. 2.6, norepinephrine (50 nM) also had no consistent effect on perfusion pressure [-1±1 mm Hg (n=18)], but the increase in $\dot{V}O_2$ induced by this agonist was totally blocked by the β-antagonist propranolol (10 μM). Neither

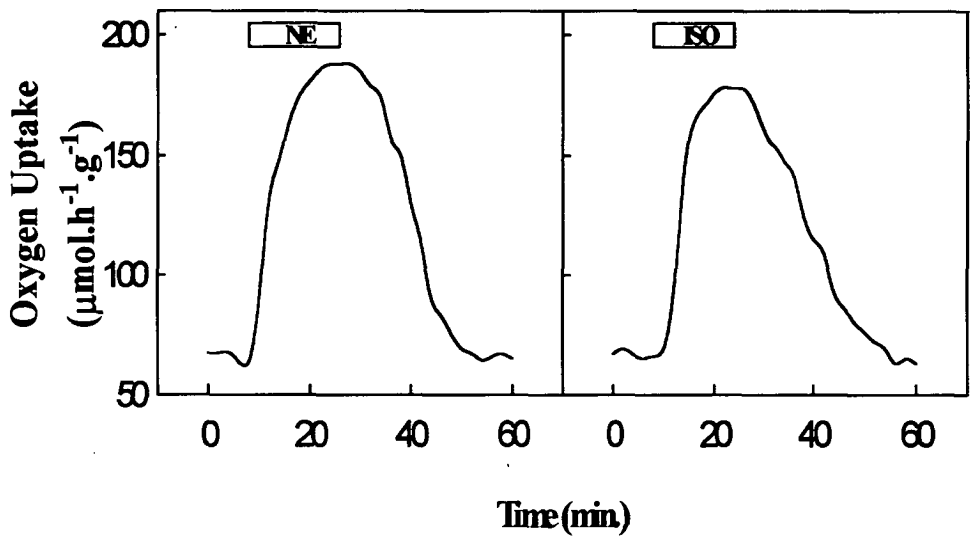


**Figure 2.4** Typical tracing of the effect of 50 nM norepinephrine (NE) and 100 nM isoproterenol (ISO) on $\dot{V}O_2$ of perfused periaortic BAT.

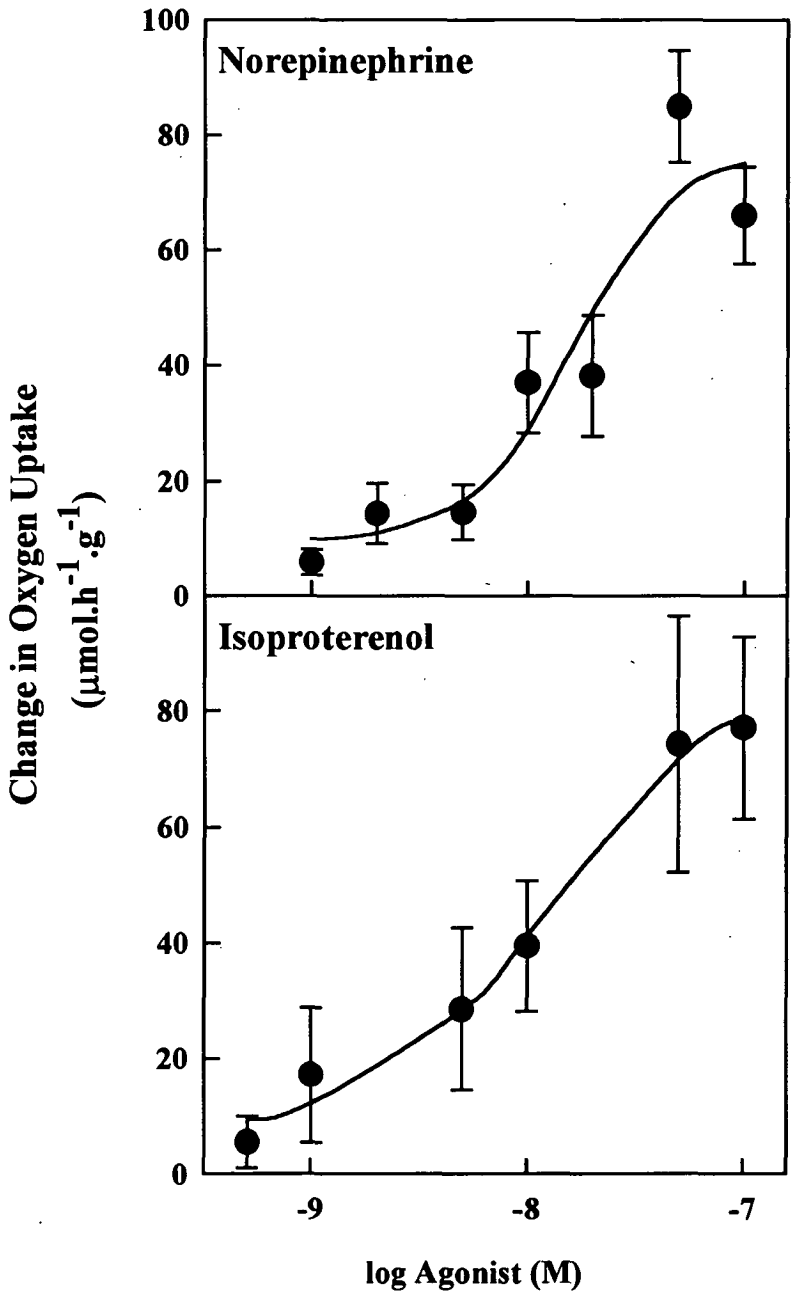


**Figure 2.5** Dose response curves for norepinephrine (NE) and isoproterenol (ISO) on change in $\dot{V}O_2$ by isolated perfused periaortic BAT. Basal $\dot{V}O_2$ was 64.3±7.4 μ mol.$h^{-1}$.g wet $wt^{-1}$ (n=53) at a flow rate of 1 ml.$min^{-1}$. Agonists were added in a step-wise manner and the preparation was allowed to fully recover at the end. Means ± SEM are shown for a minimum of 3 perfusions for each agonist.

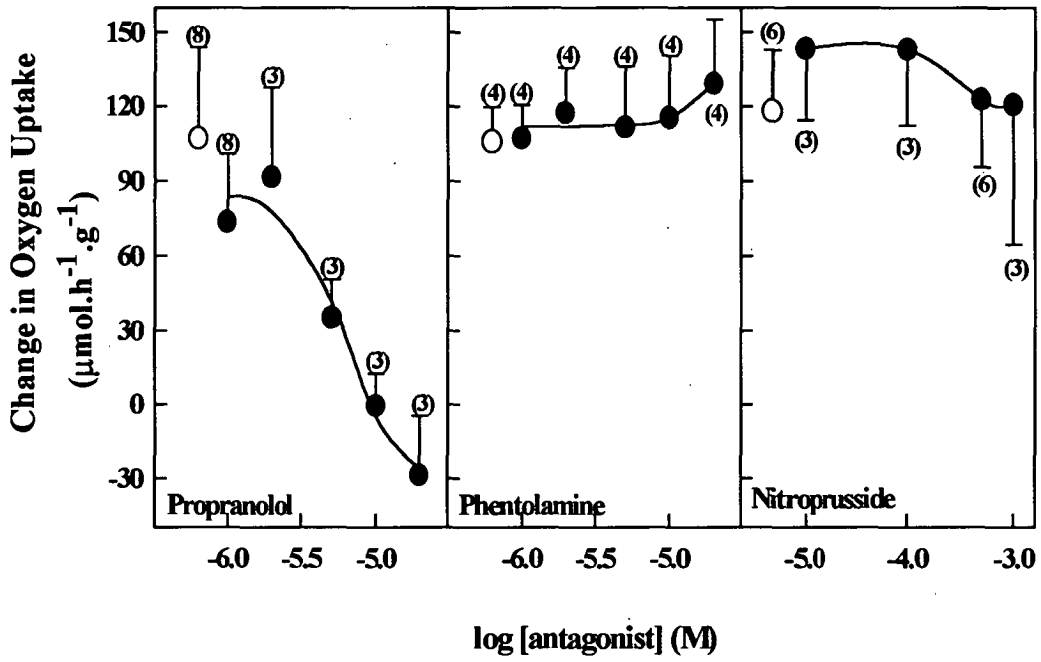


**Figure 2.6** Effects of propranolol, phentolamine and nitroprusside on norepinephrine-induced changes in $\dot{V}O_2$ of perfused periaortic BAT. Basal $\dot{V}O_2$ was 91.9±12.0 μmol.h$^{-1}$.g wet wt$^{-1}$ (n=24) and basal pressure was 66±4 mm Hg at a flow rate of 1 ml.min$^{-1}$. Agents added were 50 nM norepinephrine alone (O) or 50 nM norepinephrine plus antagonist (●). Data are shown as means ± SEM with the number of perfusions given in parentheses.

phentolamine (1-20 μM), an α-antagonist nor sodium nitroprusside (0.01-1 mM), a nitrovasodilator, had any effect on the norepinephrine-induced stimulation of $\dot{V}O_2$. In addition neither phentolamine nor sodium nitroprusside had any statistically significant effect on perfusion pressure (data not shown).

The β-agonist, isoproterenol, markedly increased $\dot{V}O_2$ and this was totally inhibited by 10 μM propranolol but unaffected by 1-20 μM phentolamine (Fig. 2.7). Half-maximal inhibition of 50 nM norepinephrine or 100 nM isoproterenol occurred at 5 μM DL-propranolol.

### 2.3.3 *Flow-induced changes in $\dot{V}O_2$ and pressure*

The relationship between flow and steady-state oxygen consumption and pressure development, as well as the effect of norepinephrine on these parameters is shown in Figure 2.8. Perfusate flow was set at 1.0, 1.4 and 1.7 ml.min$^{-1}$ for at least 15 min during which time pressure and oxygen uptake were recorded. Fig. 2.8 shows that basal $\dot{V}O_2$ (A) did not increase as the flow rate was increased, but basal perfusion pressure did. Perfusion pressure increased from 66±19 mm Hg (n=3) to 104±27 mm Hg (n=3); this was statistically significant when the data for each perfusion was treated relative to the pressure at 1.0 ml.min$^{-1}$.

Norepinephrine (50 nM) had no consistent or significant effect on pressure development at any flow rate, but maximal $\dot{V}O_2$ increased with increasing flow from 174.8±6.4 μmol.h$^{-1}$.g wet wt$^{-1}$ (n=3) at 1.0 ml.min$^{-1}$ to 236.3±27.8 μmol.h$^{-1}$.g wet wt$^{-1}$ (n=3) at 1.7 ml.min$^{-1}$. At a flow rate of 1.0 ml.min$^{-1}$, venous $PO_2$ decreased from 458 ±14 to 408±23 mm Hg (n=3), while at 1.7 ml.min$^{-1}$ it decreased from 553±15 to 480± 11 mm Hg. Thus as more oxygen was delivered, more was used, although not all of the available oxygen was removed from the perfusate and utilized by the tissue. When the flow rate was returned to 1.0 ml.min$^{-1}$ from 1.7 ml.min$^{-1}$, perfusion pressure was not significantly lower (60±17 mm Hg) than that measured originally (66±16 mm Hg). With this return to the original flow rate of 1.0 ml.min$^{-1}$, basal $\dot{V}O_2$ was significantly reduced [from 113.3±14.8 μmol.h$^{-1}$.g wet wt$^{-1}$ (n=3) to 45.6±12.6 μ mol.h$^{-1}$.g wet wt$^{-1}$ (n=3)]. The magnitude of the norepinephrine-induced increase in $\dot{V}O_2$ was the same at 1.0 ml.min$^{-1}$ both before and after the increases in flow, despite the change in basal $\dot{V}O_2$.

The dependence of the magnitude of the norepinephrine-stimulated increase in $\dot{V}O_2$ on the arterial $PO_2$ was further demonstrated with studies at 25°C. As seen in Table 2.4, at 25°C, arterial and venous (basal) $PO_2$ were significantly different from that seen at 37°C, simply due to the temperature dependent solubility of oxygen. Basal $\dot{V}O_2$ was similar at both temperatues as was the norepinephrine dose response curve. The major difference between the results gained at the two temperatures was

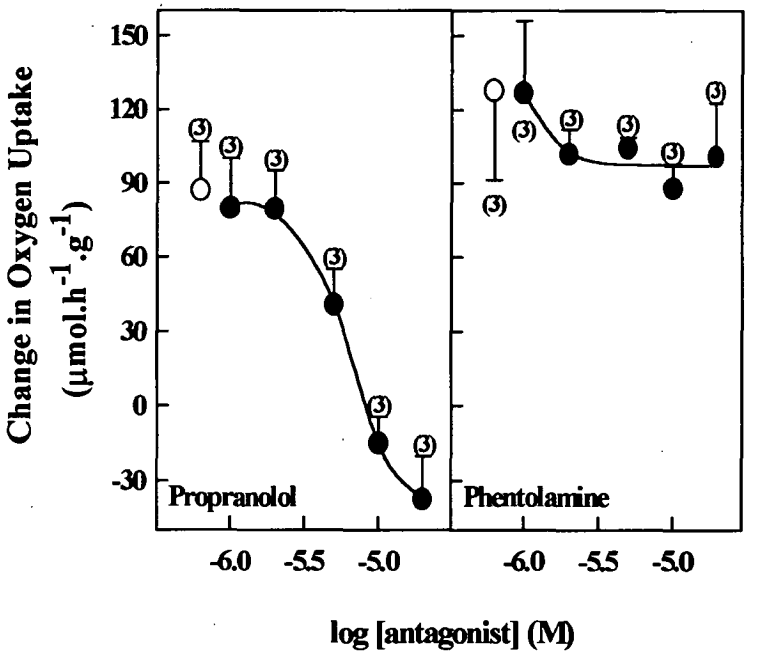


**Figure 2.7** Effects of propranolol and phentolamine on isoproterenol-induced changes in $\dot{V}O_2$ of perfused periaortic BAT. Details were as for Figure 2.6 except that agents added were 100 nM isoproterenol alone (O) or 100 nM isoproterenol plus antagonist (●).

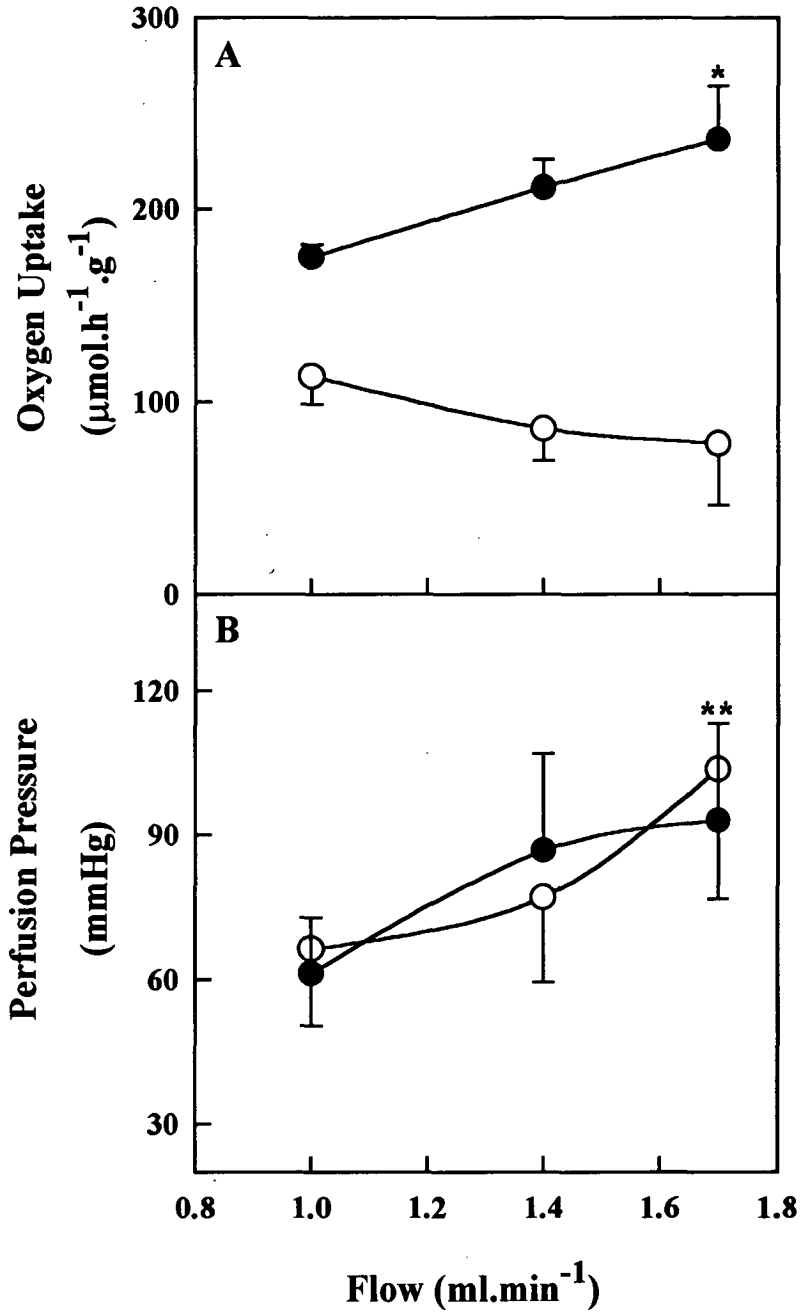


**Figure 2.8** Effect of perfusate flow and norepinephrine on $\dot{V}O_2$ (A) and pressure development (B). Data are means ± SEM for three control (O) and three norepinephrine (50 nM, ●) perfusions; bars not visible are within the symbol. *, $P<0.05$ for increase due to norepinephrine at 1.7 ml.min$^{-1}$ versus increase due to norepinephrine at 1.0 ml.min$^{-1}$. **, $P<0.05$ for the increase in basal pressure at 1.7 ml.min$^{-1}$ compared to 1 ml.min$^{-1}$.

the 50 nM norepinephrine-stimulated increase in $\dot{V}O_2$ which was significantly increased at 25°C. This is not normally the response seen when the temperature is lowered. Metabolic rate and thus oxygen consumption are normally inhibited by temperature decreases. With the same 50 nM norepinephrine-stimulated venous $PO_2$ seen at both temperatures, the data indicates that BAT stimulated $\dot{V}O_2$ is regulated with respect to the oxygen content of the blood or (perfusate).

**Table 2.4** Comparison of arterial and venous oxygen content, and the basal and 50 nM NE- stimulated $\dot{V}O_2$ of periaortic BAT perfused at 25 and 37°C.

| | | 25°C data | 37°C data |
|---|---|---|---|
| arterial $PO_2$ (mm Hg) | | 613.1±9.2* | 570.8±8.4 |
| venous $PO_2$ (mm Hg) | basal | 576.8±11.0* | 523.7±9.5 |
| | 50 nM NE | 480.0±41.7 | 449.8±13.8 |
| $\dot{V}O_2$ ($\mu mol.h^{-1}.g^{-1}$) | basal | 105.2±35.1 | 64.3±7.4 |
| | 50 nM NE | 271.0±38.4* | 148.4±11.8 |

NOTES: *, $P<0.05$; Data are means ± SEM for n=53 at 37°C and n=9 at 25°C.

## 2.4 *Discussion*

### 2.4.1 *BAT perfusion*

In this study, a perfused preparation of brown adipose tissue (BAT) from the periaortic region of the rat has been developed and characterized. BAT in the periaortic region differs very little from that found in the interscapular region, as assessed both morphologically and biochemically. This preparation was found to be responsive to norepinephrine and isoproterenol in terms of increased oxygen consumption with reversible dose-dependent effects for both agonists. The tissue also responded to the $\beta_3$ agonist, BRL 35135, with an increase in $\dot{V}O_2$.

The basal oxygen uptake rate of 64.3±7.4 $\mu mol.h^{-1}.g$ wet $wt^{-1}$ by the perfused periaortic BAT was similar to that encountered by other investigators for interscapular BAT, either as perifused tissue slices (Friedli *et al.*, 1977) or for the tissue *in vivo* (Foster and Frydman, 1978) but different from that of isolated incubated adipocytes, which was at least 4-fold greater (Bukowiecki *et al.*, 1981). Again, like perifused tissue slices of BAT, the perfused periaortic BAT preparation gave relatively low maximal oxygen uptake rates, and thus at a flow rate of 10.8 $ml.min^{-1}.g$ wet $wt^{-1}$ a maximal dose of norepinephrine or isoproterenol increased the rate to 150

$\mu mol.h^{-1}.g$ wet $wt^{-1}$. This low rate is not readily explained given that cytochrome oxidase activity is approximately 12,000 $\mu mol.h^{-1}.g$ wet $wt^{-1}$ and similar to that of interscapular BAT (Trayhurn *et al.*, 1982). There was no evidence of ischemia in the perfused periaortic BAT as the energy charge closely resembled that of freshly sampled interscapular BAT (Table 2.2). In addition, infusion of Indian ink at the end of an experiment suggested that the periaortic BAT was being homogeneously perfused (Fig. 2.5B). However, flow rate may play an important role ensuring that oxygen delivery is optimal. Evidence in support of this view comes from Fig. 2.8 which shows that although increasing the flow rate from 1.0 $ml.min^{-1}$ to 1.7 $ml.min^{-1}$ did not increase basal oxygen uptake, the stimulatory effect of norepinephrine was markedly increased such that at 1.7 $ml.min^{-1}$ (18.3 $ml.min^{-1}.g$ wet $wt^{-1}$) maximal oxygen uptake approached 240 $\mu mol.h^{-1}.g$ wet $wt^{-1}$. Even so, this value is still well below that of fully stimulated isolated brown adipocytes (2,640 $\mu mol.h^{-1}.g$ wet $wt^{-1}$; Bukowiecki *et al.*, 1981) and estimates of norepinephrine-mediated oxygen uptake for BAT *in vivo* (Foster *et al.*, 1980). Indeed these latter workers showed that for the warm acclimated rat, norepinephrine increased interscapular BAT blood flow from approx. 0.5 to 6.5 $ml.min^{-1}.g$ wet $wt^{-1}$ and the estimated oxygen uptake from approx. 50 to 2121 $\mu mol.h^{-1}.g$ wet $wt^{-1}$. However it is important to note that the oxygen consumption of the interscapular BAT *in vivo* was estimated as the product of its blood flow, the coefficient of extraction of oxygen at that flow per g of BAT, the prevailing arterial $PO_2$, and tissue weight. From these calculations it is clear that oxygen delivery is indeed a primary determinant of oxygen uptake by BAT. Blood flow rates as great as 28 $ml.min^{-1}.g$ wet $wt^{-1}$ have been reported for some depots of BAT *in vivo* (Foster, 1986). Thus to achieve maximal rates of $\dot{V}O_2$ by perfused periaortic BAT similar to those found *in vivo* or for the isolated adipocytes may require the presence of a higher arterial $PO_2$. This may be achieved by using perfusion flow rates higher than those in the present study, the addition of erythrocytes to the perfusate, or the use of whole blood as the perfusate. In the present study it appears that norepinephrine and/or oxygen availability only become limiting during maximal stimulation by norepinephrine despite venous $PO_2$ under these conditions not decreasing below 450±14 mm Hg (n=53). Whilst other factors may be involved *in vivo*, it is possible that increased flow simply improves norepinephrine and oxygen access, although this change was not apparent from dye infusion experiments.

### 2.4.2 *Is there a vascular component to BAT thermogenesis?*

Several observations in the present study suggest that vascular thermogenesis and/or vascular control of BAT thermogenesis was minimal in the constant-flow perfused periaortic BAT preparation. These include firstly, the failure of norepinephrine to consistently cause vasoconstriction or vasodilation in association with an increase in oxygen uptake (see Section 2.3.2). Thus whilst the increase in oxygen uptake occurred for all preparations, approximately half showed an increase and the other half showed a decrease in perfusion pressure. Secondly, isoproterenol mediated a large increase in oxygen uptake comparable to that mediated by norepinephrine without involving a change in pressure. Based on findings with the perfused hindlimb isoproterenol might be expected to lower pressure by dilating constricted vessels that are responsible for some of the basal oxygen uptake or that are involved in directing flow to oxygen consuming tissue. This is not unexpected in BAT as β-adrenoreceptors subserving vasodilation have not been described (Foster, 1986). Thirdly, the failure of increased flow to increase basal oxygen uptake, which may suggest that blood vessels are working maximally at the flow rate of approximately 10.8 $ml.min^{-1}.g$ wet $wt^{-1}$, or that oxygen delivery to unstimulated tissue is already optimal. Fourthly, the failure of nitroprusside or phentolamine to inhibit norepinephrine-mediated increase in oxygen uptake implies that working blood vessels do not contribute to the increased oxygen consumption and/or that norepinephrine has no site-specific effects to direct flow to oxygen consuming tissue. Fifthly, the complete inhibition by propranolol of the norepinephrine- or isoproterenol-mediated increase in $\dot{V}O_2$ indicates that thermogenesis of the perfused periaortic BAT is exclusively mediated by β-adrenergic receptors.

Overall the failure to find vascular tissue involvement in the control of BAT oxygen uptake may not be surprising. Vascular shunts have been reported to be present in interscapular BAT (Lever *et al.*, 1985; Nnodim and Lever, 1988), controlling flow into this tissue by constriction at sites on shunts putatively involving α-adrenoreceptors (Nnodim and Lever, 1988). The present findings that phentolamine failed to block the stimulatory effect of norepinephrine on oxygen uptake by the perfused periaortic BAT (Fig. 2.6) questions the direct role of α-adrenoreceptors in the control of thermogenesis or the indirect role via flow regulation within BAT. Evidence obtained by Bukowiecki *et al.* (1981) suggests that α-adrenoreceptors have little or no direct role to play as 1 μM propranolol completely blocked the increase in oxygen uptake by 100 nM norepinephrine with isolated rat brown adipocytes from the interscapular region. In addition, they showed that either dibutyryl cyclic AMP or theophylline mimicked much of the stimulatory effect of norepinephrine (Bukowiecki *et al.*, 1981). The findings of Foster (1985), who noted

that prazosin blocked norepinephrine-mediated oxygen uptake by 53% in anaesthetized cold-acclimated rats, could be explained as an alteration in the blood flow distribution external to the BAT deposits which affects thermogenesis. Thus a balance between neural input, blood flow and oxygen supply in the control of thermogenesis may occur *in vivo* but cannot be demonstrated in an *in vitro* perfusion system using infused catecholamines.

# Chapter 3

## Does skeletal muscle possess uncoupled mitochondria?

### 3.1 *General Introduction*

The mechanisms involved in thermogenesis in BAT and skeletal muscle differ greatly (Figs. 3.1 and 3.2). BAT thermogenesis involves the activation of triglyceride breakdown and both the onset and reversal of this process are slow when compared to the response elicited by norepinephrine in the perfused hindlimb. Skeletal muscle thermogenesis has been shown to be α-mediated for norepinephrine and invariably associated with vasoconstriction (Ye *et al.*, 1990; Colquhoun and Clark, 1991). As reversal occurs immediately the stimulus is removed, this process does not appear to involve an inductive process such as that known to occur in BAT.

In skeletal muscle, the norepinephrine-stimulated increase in $\dot{V}O_2$ has been linked with the action of vasoconstriction rather than with a specific receptor mediated effect as other vasoconstrictors such as vasopressin and angiotensin II (Colquhoun *et* al., 1988) are also able to cause an effect similar to that for NE despite these vasoconstrictors being thought to act via different receptors. Alleviation of the vasoconstriction for any of these agonists (using α-antagonists, nitrovasodilators or β-agonists) removes the stimulated increases in $\dot{V}O_2$. The β-antagonist propranolol (the DL form at 10μM) has no effect on the magnitude of either the agonist-induced increases in pressure or oxygen uptake, indicating that the response is not due to activation of β-adrenoreceptors. Such findings effectively divorce this response from that seen in BAT and remove the possibility of BAT presence in the skeletal muscle of the hindlimb that is being perfused. Similar responses to that seen in the perfused rat hindlimb have also been found in perfused limbs from a marsupial (the rat-kangaroo *Bettongia gaimardi* - Ye *et al.*, 1995) and a bird (chickens - Appendix 1).

The evidence linking the increases in resting skeletal muscle $\dot{V}O_2$ to vasoconstriction appears strong but the mechanism by which this thermogenic response occurs is still not understood.

Increases in $\dot{V}O_2$ by a tissue indicate that one of two things is occurring. The first is that ATP hydrolysis has been increased and hence resynthesis has also been accelerated. This results in an increased consumption of oxygen by the mitochondria as they maintain their membrane potential despite the activation of the $F_0F_1$-ATPase proton channel with the phosphorylation of the ADP. The second possibility is that

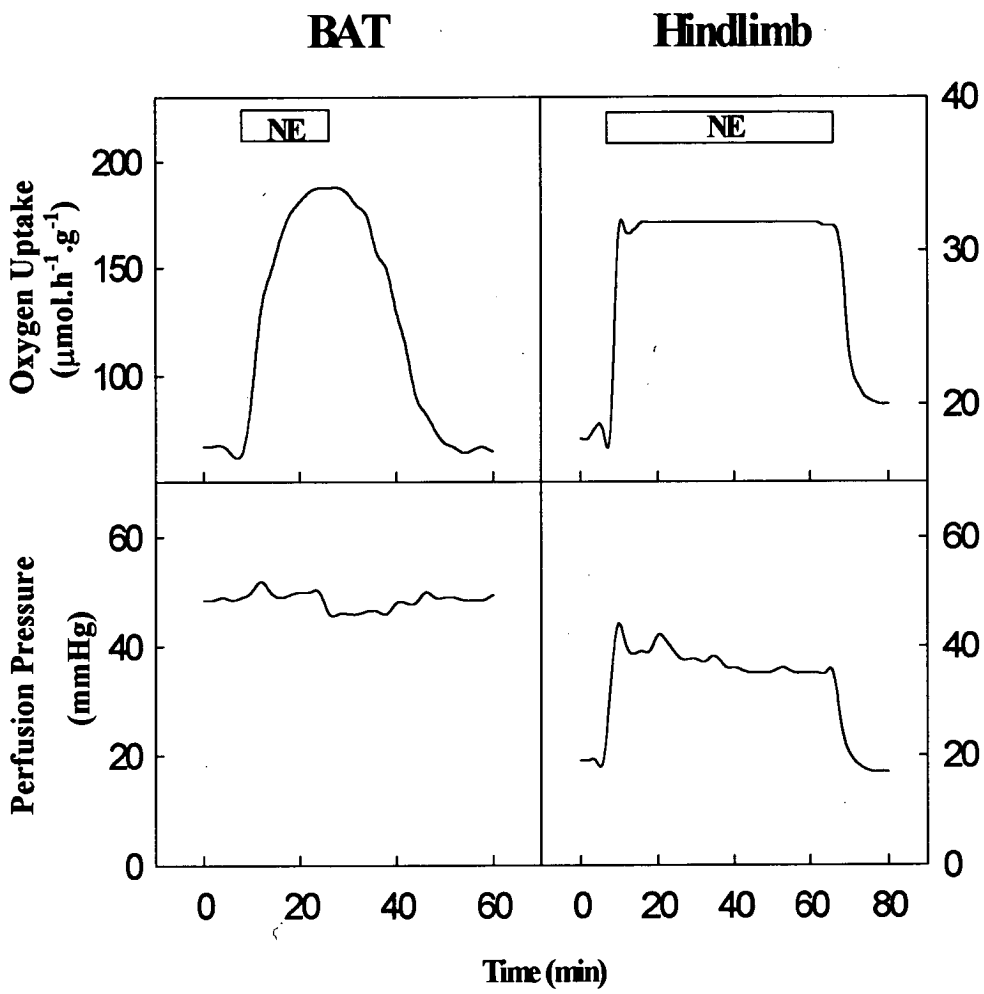


**Figure 3.1** Typical traces of norepinephrine-induced changes in oxygen uptake and perfusion pressure in perfused periaortic BAT and hindlimb at 37°C using a Krebs-ringer bicarbonate buffer as described in Section 2.2.1 (1% BSA for BAT and 4% BSA for hindlimb). (Hindlimb data from S. Rattigan 1994. personal communication).

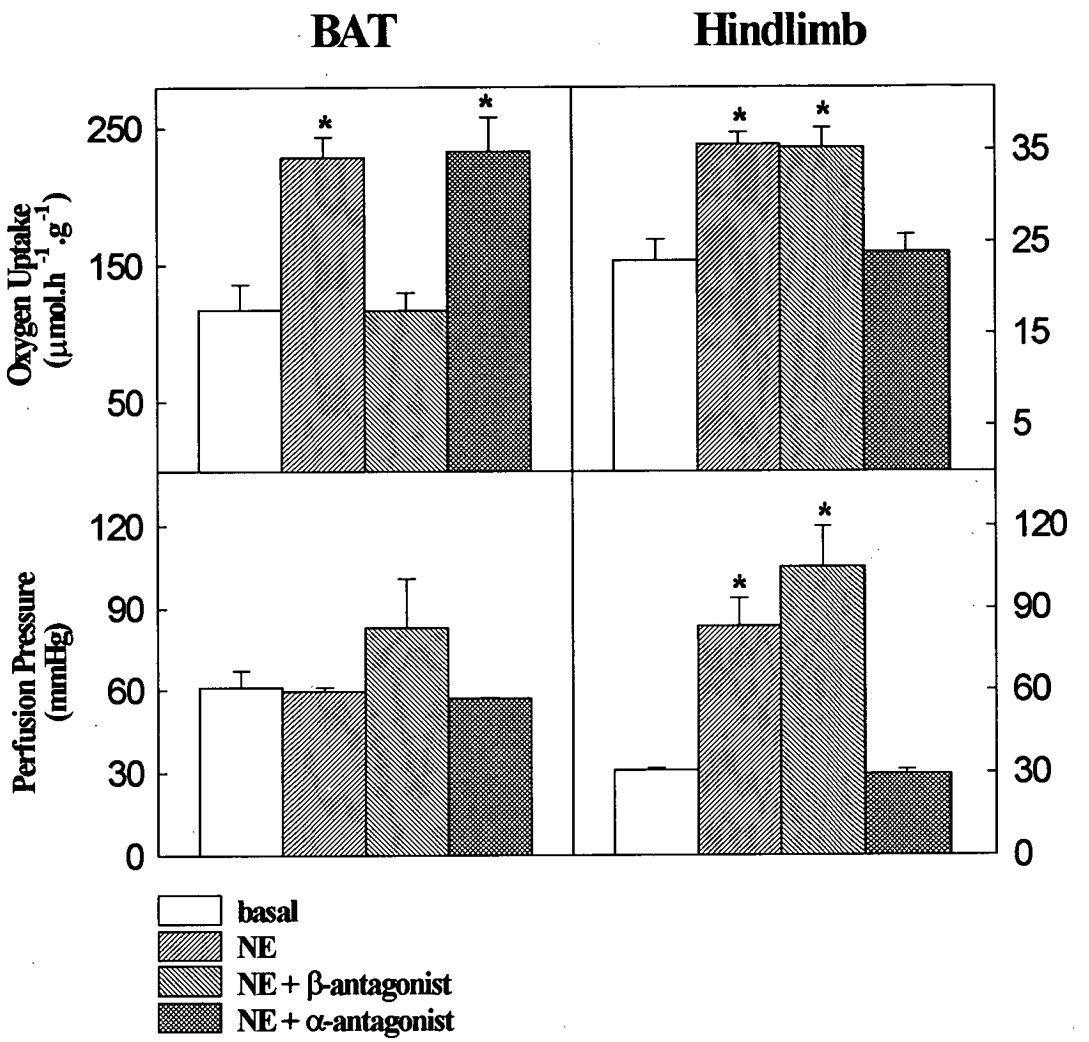


**Figure 3.2** Histogram showing the different effects of α- and β-antagonists on the norepinephrine-induced increases in oxygen uptake and perfusion pressure in perfused periaortic BAT and perfused hindlimb. Norepinephrine was infused at 100 nM in BAT and 50 nM in the hindlimb. The β-antagonist used was propranolol (DL at 10 μM). The α-antagonist in BAT was phentolamine (10 μM) and in the hindlimb was prazosin (1 μM). Perfusion flow rates were 4 and 1 $ml.min^{-1}$ for the hindlimb and BAT preparations respectively. *, $P < 0.05$ when compared to basal conditions. ($n > 3$) (Hindlimb data from S. Rattigan 1994. personal communication).

mitochondrial uncoupling, such as that present in BAT, has been stimulated, causing an increase in respiration but not ADP phosphorylation.

Three hypotheses have been generated in an attempt to explain the increases seen in the perfused hindlimb with the addition of various agonists, although only two attempt to explain the apparent link between vasoconstriction and the induced $\dot{V}O_2$.

Hypothesis 1. Vasoconstrictors act directly on the skeletal muscle to cause an increase in $\dot{V}O_2$. Evidence against this hypothesis is fairly strong. The response is not receptor specific as norepinephrine, vasopressin and angiotensin II, which all act via different receptors, all elicit similar responses. The response is absent when norepinephrine is added to perifused mouse (Dubois-Ferriere and Chinet, 1981) or rat (Hettiarachchi *et al.*, 1992) skeletal muscle. This lack of a response to a vasoconstrictor when added to the perifused muscle indicates the essential role that the vascular system plays in the response elicited in the perfused resting skeletal muscle system. The predominant receptors on skeletal muscle cells are β while those found predominating on the arterioles are α (Martin *et al.*, 1990). As the effect of norepinephrine is α- and not β-mediated, a direct skeletal muscle effect becomes unlikely.

Hypothesis 2. The increase in $\dot{V}O_2$ produced by the addition of a vasoconstrictor to the perfused rat hindlimb is due to the work done by the vasculature as it contracts to increase, and maintain the increase in perfusion pressure (Colquhoun and Clark, 1991 and references therein). On removal of the stimulus both the oxygen and pressure return to basal levels as the vasculature dilates and ATP usage is reduced to basal levels. Supporting this hypothesis are experiments using the isolated perfused rat mesenteric artery arcade (Dora *et al.*, 1991; Ye *et al.*, 1990a). In this preparation, the authors found that vasoconstrictors (norepinephrine, vasopressin and serotonin) induced increases in both perfusion pressure and $\dot{V}O_2$. Opposing this idea of a direct relationship between increases in perfusion pressure and $\dot{V}O_2$ are data obtained with the infusion of serotonin into the hindlimb. In this tissue, serotonin causes vasoconstriction but inhibits basal $\dot{V}O_2$ (Dora *et al.*, 1991). High concentrations of infused norepinephrine (10μM) have also been found to inhibit oxygen consumption even though they still increase the perfusion pressure (Dora *et al.*, 1991). These data, together with that obtained in BAT, a highly vascularized tissue in which increases in $\dot{V}O_2$ were shown to occur in the absence of any vasoconstriction (Chapter 2), suggest that it is unlikely that the vasculature is actively contributing to the norepinephrine-stimulated increases in $\dot{V}O_2$ in the hindlimb.

Hypothesis 3. This hypothesis is similar to Hypothesis 2 in that it has an absolute requirement for the vasculature in the generation of the increases in $\dot{V}O_2$. The increases in perfusion pressure cause a redistribution of perfusate flow. This would involve functional vascular shunts and the control of the proportion of flow regulated by site-specific vasomodulators (Dora *et al.*, 1991) as postulated in Figure 3.3. This could explain both the increases and decreases in $\dot{V}O_2$ seen when different agonists or concentrations of agonists are used. In essence, under basal conditions, a greater proportion of the perfusate flow is through the non-nutritive capillaries. When a Type A vasoconstrictor (one that causes an increase in $\dot{V}O_2$) is added to the perfused rat hindlimb, the proportion of flow through the non-nutritive capillaries decreases and that through the nutritive capillaries increases. The opposite occurs when a Type B vasoconstrictor (one that causes a decrease in $\dot{V}O_2$) is added, with the proportion of perfusate flow through the nutritive capillaries decreasing even further. These changes in the proportion of perfusate flow to nutritive and non-nutritive capillaries are then somehow responsible for the alterations in the tissue's oxygen usage. This flow redistribution may increase $\dot{V}O_2$ by:

(a) directing perfusate flow to previously hypoxic areas. Two pieces of data negate this possibility. The first is that the induced oxygen response remains for as long as the stimulus is present, and the second is that the adenine nucleotide levels and the energy charge (Table 3.1) are not significantly different from those found in hindlimb muscle *in vivo*.

**Table 3.1** Comparison of high energy phosphate concentrations in the muscles from the perfused rat hindlimb with those present *in vivo*. The soleus-plantaris-gastrocnemius muscle group was examined (J-M. Ye, 1994 personal communication).

| | Metabolite concentration (μmol/g dry weight) | | | | |
|---|---|---|---|---|---|
| | ATP | ADP | AMP | ΣAd | energy charge |
| in vivo | 28.18±1.13 | 2.69±0.21 | 0.23±0.03 | 31.10±1.34 | 0.95±0.01 |
| basal perfusion | 28.05±0.46 | 2.04±0.13 | 0.22±0.03 | 30.30±0.44 | 0.96±0.00 |

(b) a direct response to a change in the rate of supply of nutrients and removal of products from specific muscle regions, and thus stimulation of muscle $\dot{V}O_2$.

or (c) the stimulation of the production of a signal substance released by the vascular tissue that affects skeletal muscle oxygen consumption. This may include

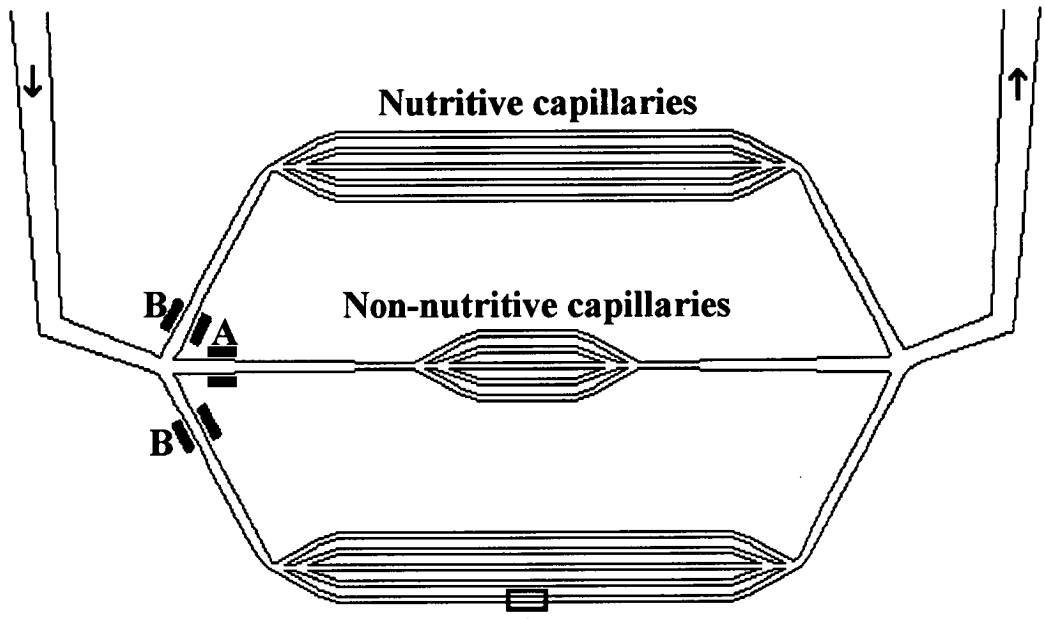


**Figure 3.3** Proposed relationship between vasculature and skeletal muscle. A, sites for Type A vasoconstrictors; B, sites for Type B vasconstrictors. The arrows indicate the direction of blood flow. (Adapted from Clark *et al.*, 1995). Enlargement (rectangle) is further elaborated in Figure 3.4.

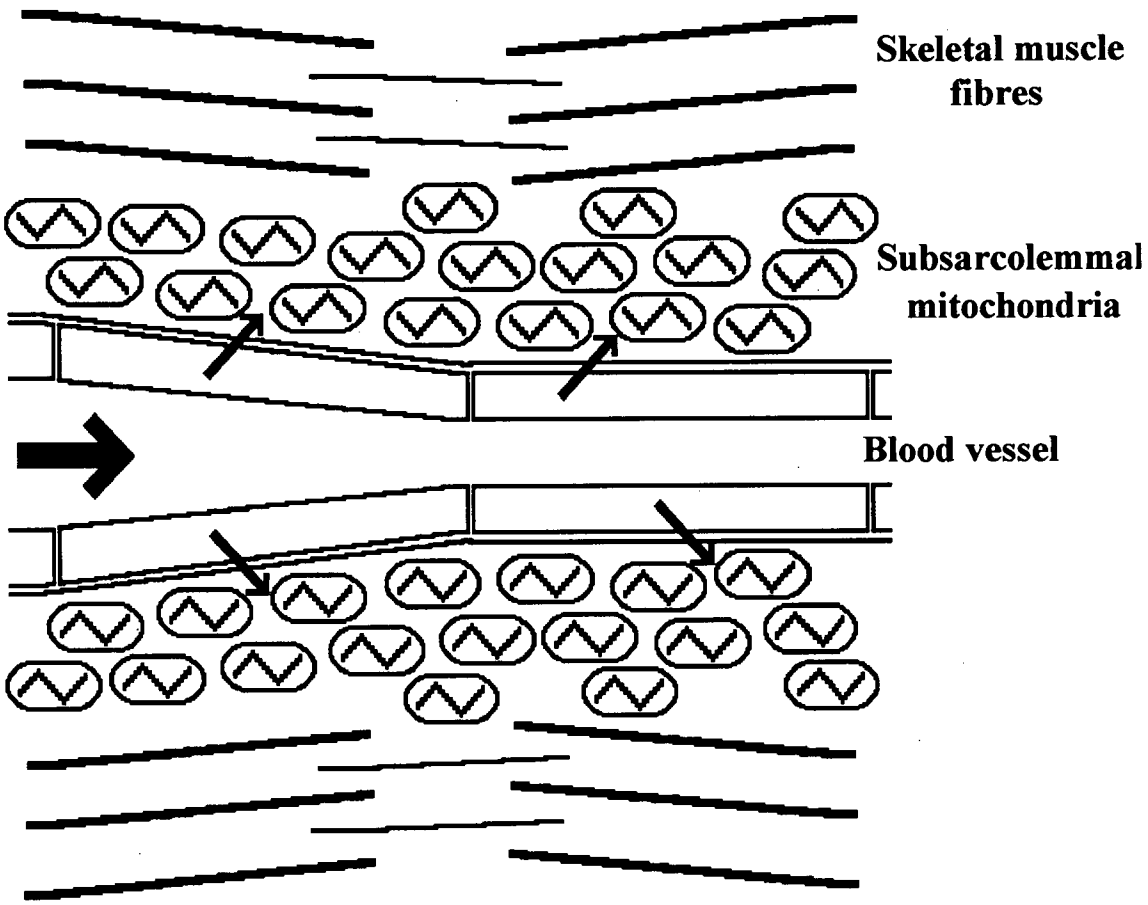


**Figure 3.4** Diagramatic enlargement of the area given in Figure 3.3, indicating the possibility of a flow-induced release of a paracrine substance from the vasculature that has a direct effect on the skeletal muscle and its thermogenic output. This substance may be released due to the vasoconstriction-induced flow redistribution to nutritive vessels.

the production of an uncoupler, possibly derived from the vasculature (Fig. 3.4), that will allow thermogenesis to occur in skeletal muscle in a manner similar to that known to occur in BAT.

Published data (Clark *et al.*, 1995, and references therein) have shown that resting muscle thermogenesis is controlled by vasomodulation and blood flow. The vasculature appears to regulate hindlimb, or skeletal muscle, thermogenesis but does not itself directly contribute to the agonist-induced changes in $\dot{V}O_2$.

In skeletal muscle, the nutritive capillaries are thought to wind around the muscle fibres (Potter and Groom, 1983; Harrison *et al.*, 1990). Adjacent to these capillaries are found a subpopulation of skeletal mitochondria - the subsarcolemmal (SS). The other skeletal muscle mitochondrial population is the intermyofibrillar (IMF) and these are located further from the capillaries between the muscle fibrils. If vasoconstrictors are able to alter the proportion of blood flow to these nutritive capillaries, and the vessels themselves are not responsible for the increases seen in the oxygen uptake of the perfused tissue, then the skeletal muscle mitochondria may be responsible.

### 3.1.1 *Skeletal muscle mitochondria*

Muscle mitochondria have been shown to be located in two distinct regions in the cells - just beneath the sarcolemma are the SS population, and between the myofibrils are located the IMF population. In cardiac muscle, differences between these two populations have been characterized (Palmer *et al.*, 1977; 1981; 1984), with the IMF mitochondria exhibiting higher inner membrane and matrix enzyme activities than the SS population. State III or ADP-stimulated respiration is almost 50% greater in the IMF mitochondria than in the SS (Palmer *et al.*, 1977). Recent work (Cogswell *et al.*, 1993) demonstrated that similar differences are also present in the two populations of mitochondria from rat skeletal muscle. IMF mitochondria had higher state III respiration, higher cytochrome-c oxidase activity, lower succinate dehydrogenase activity, lower cardiolipin content, and similar cytochrome *a*, *b*, *c* and $c_1$ content to the SS population.

These differences between the two populations of mitochondria in skeletal muscle may suggest that they have different functions related to metabolic compartmentation. One population may be more responsible for the apparent thermogenic action of skeletal muscle than the other. Due in part simply to the anatomical location of the mitochondria, one population (probably the SS) could be either permanently uncoupled or capable of being uncoupled whereas the other is not.

### 3.1.2 *Mechanism of resting skeletal muscle thermogenesis*

The contribution of skeletal muscle to facultative thermogenesis appears to be approximately 2 Watts.kg$^{-1}$ (Colquhoun and Clark, 1991). This was calculated from the data of Grubb and Folk (1976) where an increase of 0.26 μmol oxygen uptake.g hindlimb$^{-1}$.min$^{-1}$ was induced by a maximal dose of NE in rats acclimated to 22°C, assuming a standard average energy value of 4.83 kcal.l$^{-1}$ of oxygen at STP (1 kcal = 4.8155 kJ). Using the same assumptions, BAT is capable of generating at least 259 Watts.kg$^{-1}$ (Girardier and Stock, 1983). In a warm-acclimated rat where BAT is 0.8% (Foster and Frydman, 1979) and skeletal muscle is 43% of body mass, skeletal muscle would be able to contribute nearly half of the maximal possible thermogenic contribution of BAT. With a possible contribution by skeletal muscle of this magnitude to whole body thermogenesis, the mechanism involved in this process is therefore of interest.

As nature tends to be fairly conservative, in that if something is found to work once, it is usually repeated in other systems it was postulated that if skeletal muscle possessed a thermogenic mechanism it could possibly be similar to that found in brown adipose tissue. In brown adipose tissue, purine (di- and triphosphate) nucleotides are responsible for the inhibition of the uncoupling protein and this protein belongs to a family of mitochondrial transporters (Klaus *et al.*, 1991; Klingenberg 1990; Walker and Runswick, 1993). Thus if a similar thermogenic mechanism exists in another tissue (*e.g.* skeletal muscle), it is possible that ATP, fatty acids and/or one of this family of mitochondrial membrane proteins may be involved in the process.

## 3.2 *ATP as a mediator of skeletal muscle thermogenesis*

### 3.2.1 *Introduction - A role for ATP in the maintenance of homeothermy*

BAT thermogenesis has been shown to be regulated by purine nucleotides and as such the possibility of a nucleotide regulated thermogenic mechanism in skeletal muscle must be examined.

The structural similarity of other mitochondrial membrane proteins with the uncoupling protein of BAT suggests the possibility that one or more of them may be able to contribute to a thermogenic mechanism if required. If such a mechanism is present in other tissues, the suggestion that it is regulated by purine nucleotides (the regulatory substances of BAT) is also feasible.

Rather than having an inhibitory effect, a direct role for ATP in activating thermogenesis was suggested by the finding of a novel proton-translocating pathway in yeast mitochondria (Prieto *et al.*, 1992). ATP was found to induce both oxygen consumption and proton dependent swelling in a dose-dependent manner.

With the report of this ATP-regulated proton conductance pathway in yeast mitochondria, skeletal muscle SS mitochondria were examined for the presence of a similar mechanism. Proton permeability as well as respiratory rate were measured on addition of ATP to assess the capability of this nucleotide to cause uncoupling in these mitochondria.

#### 3.2.1.1 *The mitochondrial transport protein superfamily*

These proteins have been shown to have considerable sequence homology with the uncoupling protein of BAT (Klaus *et al.*, 1991; Klingenberg 1990; Walker and Runswick, 1993). Molecular weights are in the vicinity of 28 to 32 k daltons (Klingenberg, 1990) and include the ADP/ATP, phosphate and oxoglutarate/malate carrier proteins that are found in the inner membranes of mitochondria. These all appear to belong to the same protein superfamily, with sequences approximately 300 amino acids long and characterized by the presence of a threefold sequence repeat of about 100 amino acids (Klaus *et al.*, 1991; Walker and Runswick, 1993 and references therein). These tandem repeats from these different proteins are interrelated and probably have similar secondary structures. All of the members of this carrier superfamily of characterized specificity are confined to mitochondria and none of them have been detected in any other subcellular compartment.

The tissue distribution of the carrier proteins, and their isoforms when present, differ. The amount of a carrier protein or the isoform type present in a mitochondrial

population could have a major influence on the respiratory or thermogenic performance of the tissue. An example of this is the uncoupling protein, which is only found in BAT, and which accounts for the marked thermogenesis of this tissue.

Other mitochondrial carriers have been biochemically characterized in mammalian mitochondria (carriers for the exchange of pyruvate for $OH^-$ and acyl-carnitine for carnitine, carriers for citrate, glutamate, ornithine and dicarboxylate) and it is likely that they will also belong to this carrier protein superfamily. They possess similar molecular weights and tend to copurify with them, but until their sequences have been determined and compared to those of the known superfamily members it will remain uncertain as to whether or not they belong (Walker and Runswick, 1993 and references therein).

### 3.2.1.2 *Proton permeability*

Rates of solute transport across mitochondrial membranes can be assessed using a swelling technique. The amount of light scattered by a mitochondrial suspension depends on the matrix volume and has been shown to depend, in a complex but predictable manner, on native structure of the mitochondrion (Beavis *et al.* 1985). Matrix swelling causes a decrease in the intensity of light scattered by the suspension.

The transport of solutes across the inner membrane of mitochondria is accompanied by transport of water. This causes swelling or contraction of the matrix compartment depending on the influx or efflux of both the solutes and the water. The permeation of water is sufficiently rapid to assure the maintenance of the osmotic equilibrium of the matrix and that the rate of swelling is limited by the rate of solute transport (Beavis *et al.*, 1985). This technique is capable of providing quantitative information about solute transport across the inner membrane (Garlid and Beavis, 1985).

Lipophilic weak acids and bases are both thought to cross the lipid bilayer of the inner mitochondrial membrane via non-ionic or electroneutral diffusion (Fig. 3.5). The mitochondria swell when suspended in salt medium provided pathways exist for both anion and cation transport. In the presence of potassium salts, valinomycin (val), a potassium ionophore, is used to induce K+ transport into the matrix and an uncoupler (FCCP) is used to allow H+ efflux to the cytosol. Net salt transport then occurs if an electroneutral pathway for net acid transport is present (Garlid and Beavis, 1985). The swelling of the mitochondria in the presence of valinomycin is in fact limited by the rate at which protons entering the matrix with acetate can leave in order to maintain charge balance.

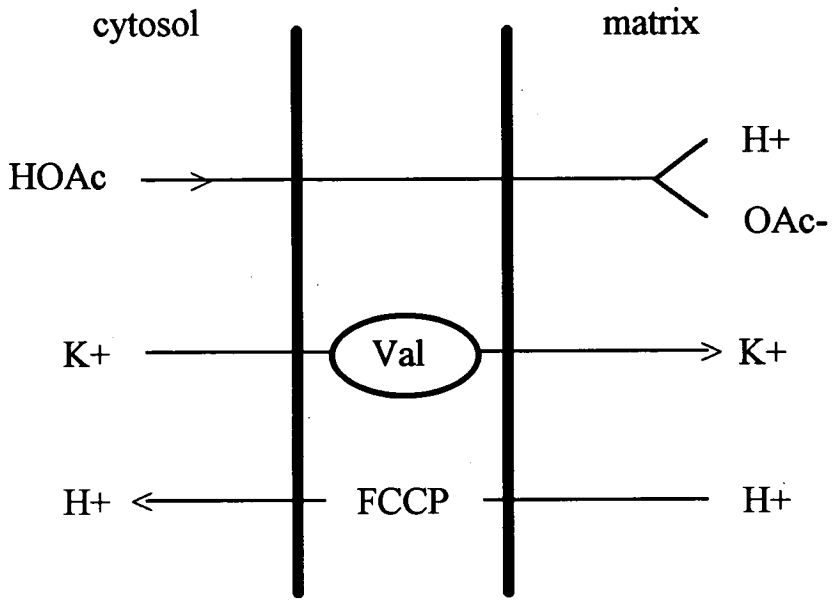


**Figure 3.5** Diagramatic representation of the movement of ions across the mitochondrial membrane during swelling. Adapted from Brierley *et al.* (1978) and Garlid and Beavis (1986).

By measuring the swelling rate of mitochondria in response to various compounds, any increases in the rate of swelling would be indicative of an increase in proton permeability and hence uncoupling. Prieto *et al.* (1992) used liver and BAT mitochondria as both a control and a comparison to the yeast mitochondria. They found that liver mitochondria exhibited very slow swelling rates unless an exogenous uncoupler was present, whereas BAT mitochondria swelled rapidly unless an inhibitor of the uncoupling protein (*e.g.* ATP) was present (Prieto *et al.*, 1992). In this study, liver mitochondria which are generally very robust have also been used as a control and comparison for skeletal muscle mitochondria.

### 3.2.1.3 *Mitochondrial respiration*

For mitochondria to be able to form ATP, which appears to be their primary function, a proton gradient across the inner mitochondrial membrane is necessary. Several protein complexes are involved in the process (the electron transport chain) that the mitochondria use to form the proton gradient. These are known as NADH-ubiquinone reductase, succinate-ubiquinone reductase, ubiquinone-cytochrome c reductase and cytochrome c oxidase or as Complexes I to IV. As electrons flow from one complex to the next, protons are translocated across the inner mitochondrial membrane (Fig. 3.6).

Complex I catalyzes the oxidation of NADH, with the transfer of two electrons from NADH to ubiquinone. It also pumps three protons from the mitochondrial matrix into the intermembrane space so that the energy released by the redox reaction is conserved in the form of the proton gradient. Complex II also catalyzes the reduction of ubiquinone but here, the electron donor is $FADH_2$ and no protons are pumped across the mitochondrial membrane. Complex III is then reduced by the electrons that are passed on from the ubiquinone with four protons thought to be pumped out of the mitochondrial matrix. In turn, Complex III passes the electrons onto Complex IV which is the terminal component of the electron transport chain. Here, the four electron reduction of $O_2$ to water occurs and two protons are pumped into the intermembrane space. Nearly 90% of cellullar oxygen is used by this terminal complex of the electron transport chain (Rawn, 1989).

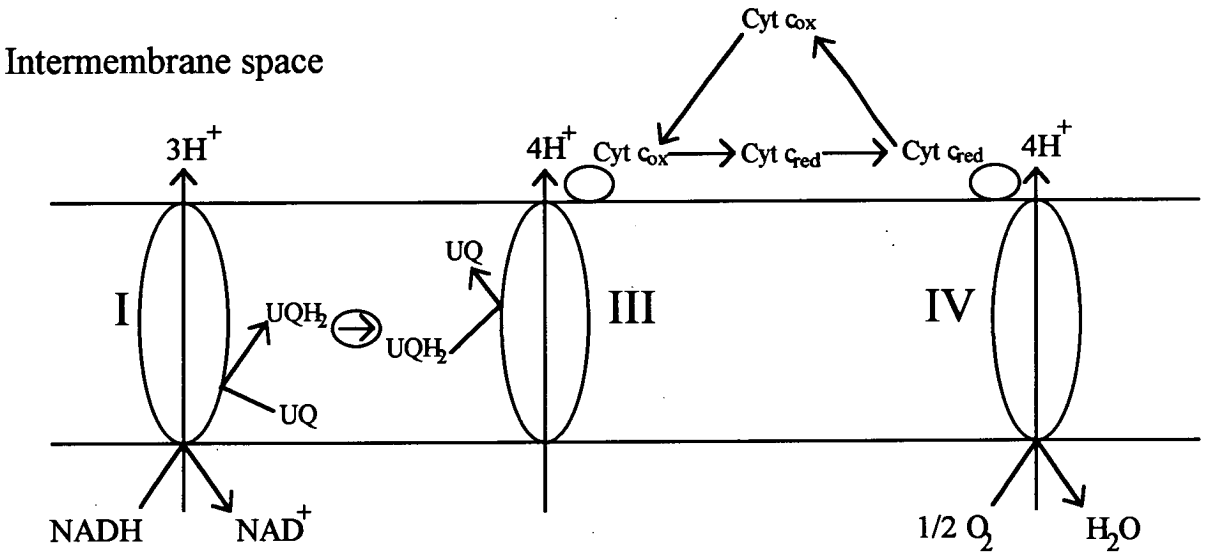


**Figure 3.6** Mitochondrial proton transport. Diagramatic representation of the movement of electrons and protons through the electron transport chain and across the inner mitochondrial membrane respectively. UQ = ubiquinone; UQH2 = ubiquinol; Cyt c = cytochrome c; ox = oxidized; red = reduced; I = Complex I; III = Complex III; IV = Complex IV. Adapted from Rawn (1989).

The rate of electron transport is tightly regulated, with the NADH/NAD (and $FADH_2$/FAD) ratio, the partial pressure of oxygen and the pH gradient all contributing to the rate control. On the addition of an uncoupler (such as FCCP or PCP) , the proton gradient in respiring mitochondria is dissipated, as the uncoupler allows diffusion of protons back into the mitochondrial matrix (Hanstein, 1976 and Rottenberg, 1990 and references therein). Then, although all necessary substrates may be present, ATP production will not occur. The oxidation reactions of the electron transport chain will however continue at a maximal rate. Energy is thus released as heat rather than in the form of ATP.

One way of measuring the movement of electrons through the electron transport chain is to monitor the rate at which the mitochondria will consume oxygen as its conversion to water is the terminal step. These respiratory rates can be assessed using an electrode for constant measurement of the oxygen tension of the respiratory medium. The rates at which oxygen is consumed by the isolated mitochondria under various conditions can then be calculated.

### 3.2.2 *Methods*

#### 3.2.2.1 *Rat skeletal muscle subsarcolemmal mitochondria isolation*

Rats were anaesthetized as given in section 2.2.1. The protocol followed was essentially that of Balon *et al.* (1992). Briefly, muscle from both hindlimbs was stripped from the bones, fat, skin and tendons, and washed in 150 mM KCl. The tissue was then minced with scissors in the isolation buffer (100 mM sucrose, 100 mM KCl, 7 mM EDTA, 0.2% FFA BSA, 330 U/ml heparin and 50 mM Tris-maleate pH 7.4), and homogenized using a Potter-Elvehjem homogenizer. Heparin was added to prevent the formation of a gelatinous consistency and hence mitochondrial agglutination during homogenization (Nedergaard and Cannon, 1979). It has also been shown that when skeletal muscle mitochondria are isolated in the presence of heparin, they exhibit very tight coupling and high respiratory activity as compared to those isolated in its absence (Dow, 1967). The homogenate was centrifuged at 800 g for 10 minutes, and the supernatant recentrifuged at 14000 g for 10 minutes. The "buffy coat" was removed from the surface of the pellet and the mitochondria washed twice with the isolation buffer and recentrifuged at 7500 g for 10 minutes. The final pellet was resuspended in 2 ml of the isolation buffer and stored on ice.

### 3.2.2.2 *Liver mitochondria isolation*

Rats were anaesthetized as described in section 2.2.1. The protocol for mitochondrial isolation was essentially that of Schneider (1948). Briefly, the liver was quickly removed and washed in ice-cold isolation buffer (250 mM sucrose, 1 mM EDTA, 0.2% FFA BSA and 10 mM Tris-maleate pH 7.4). Approximately 2 g of liver was homogenized in 20 ml of the isolation buffer using a Potter-Elvehjem homogenizer, for 1 minute at 500 rpm. The homogenate was then layered onto 10 ml of 0.34 M sucrose in 2 x 40 ml polyethylene centrifuge tubes, to form two distinct layers, and centrifuged at 800 g for 10 minutes. The supernatant or "top layer" was then removed and recentrifuged at 8000 g for 10 minutes, and the resulting pellet washed with isolation buffer prior to centrifugation at 8000 g for 10 minutes again. The mitochondrial pellet was then resuspended in 2 ml of the isolation buffer and stored on ice.

### 3.2.2.3 *Protein determination - Lowry method*

An adaptation of the method described by Lowry *et al.* (1951) was used for the determination of the protein content of the rat, both liver and skeletal muscle, mitochondrial suspensions. An aliquot of the mitochondrial suspension (10-100 µl) was added to 250 µl of 10% TCA, mixed and stored on ice for 20 minutes before being centrifuged at 8000 g for 10 minutes. The pellet was dissolved in 2.5 ml buffer (2% sodium carbonate in 0.1 N sodium hydroxide, containing 0.01% copper sulphate and 0.02% potassium tartrate). Absorbance at 660 nm was determined both before and after the addition of 250 ml of 50% Folin's reagent (after a 20 minute incubation period at room temperature). BSA (fraction V from Sigma) was used as the protein standard.

### 3.2.2.4 *Proton permeability - Swelling assays*

Rates of proton movement through the inner mitochondrial membrane were determined at 37°C in the presence of potassium acetate and valinomycin (Nicholls and Rial, 1989). Proton permeability was measured by following the rate of osmotic swelling of the mitochondria and measured as a decrease in the amount of light scattered. Measurements were made at an angle of 90° to the incident light using a Cary spectrophotometer. Cuvettes were continuously stirred. The incubation media consisted of 0.1 M potassium acetate, 2 mM magnesium chloride, 12.1 $\mu g.ml^{-1}$ oligomycin, 7 µM atractyloside, 0.5 $\mu g.ml^{-1}$ antimycin A and 10 mM Tris-maleate pH 7.4. Protein concentration was 0.2 $mg.ml^{-1}$. Basal proton permeability was calculated from the rate of swelling ($\Delta OD.min^{-1}$) at 526 nm after the addition of 1.2 µM

valinomycin. Maximal rates were then calculated after the further addition of test compounds.

### 3.2.2.5 *Respiration*

Mitochondria were incubated at 37°C in a Clark type oxygen electrode.

For muscle mitochondria, the respiration medium contained 45 mM sucrose, 10 mM mannitol, 15 mM KCl, 7 mM EDTA, 2 mM $MgCl_2$, 6 mM $KH_2PO_4$ 0.2% fatty acid free BSA and 25 mM Tris-maleate pH 7.4. Pyruvate (5 mM) + malate (5 mM) were the added substrates.

For liver mitochondria, the respiration medium contained 45mM sucrose, 10 mM mannitol, 15 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 6 mM $KH_2PO_4$ 0.2% fatty acid free BSA and 10 mM Tris-maleate pH 7.4. Succinate (5 mM) + rotenone (5 μM) was the substrate used with liver mitochondria.

All media were air equilibrated. Concentrations of oligomycin and atractylate were as given for the swelling assays (Section 3.2.2.4).

## 3.2.3 *Results*

### 3.2.3.1 *Swelling assays*

SS skeletal muscle mitochondria were found to swell on addition of ATP in a dose-dependent manner (Fig. 3.7). The maximum swelling rate at 3.62 mM ATP of $0.588 \pm 0.141$ U.mg protein$^{-1}$ was not statistically different ($p>0.05$) from the $0.782 \pm 0.209$ U.mg protein$^{-1}$ encountered on addition of 40 μM FCCP. Other di- and tri-nucleotides (guanosine, inosine, cytosine, uridine) were not able to mimic this ATP effect. Table 3.2 shows that maximum physiological concentrations of creatine, phosphate and ADP encountered with exhaustive steady-state isometric contraction in the rat hindlimb *in vivo* (Brindle *et al.*, 1989) had no effect on the ATP-induced swelling rate. ADP at higher concentrations (0.75 mM ADP) however, did have an inhibitory effect on the swelling induced by ATP (0.75 mM).

ATP analogues (ATP-γ-S, AMP-PNP and AMP-PCP) were also examined. From Dawson *et al.*, (1986) descriptions of their modes of action were obtained. ATP-γ-S or adenosine 5'-O-(-3-thiotriphosphate) is able to be translocated by the adenine nucleotide translocator (ANT), but is a potent inhibitor of ATP-driven reverse electron transport. AMP-PNP or 5' adenylylimidodiphosphate is not able to be hydrolysed by ATPases. AMP-PCP or adenylylmethylenediphosphate is resistant to phosphorylation and transphosphorylation reactions, and is able to replace ATP in the atractyloside-sensitive ANT. None of these analogues, when added alone,

induced mitochondrial swelling, but ATP-γ-S and AMP-PNP were able to completely inhibit the ATP-induced swelling effect when added at equimolar concentrations. The effect of other mitochondrial inhibitors was also investigated and it was found that although cyanide did not adversely affect the ATP-induced rate of mitochondrial swelling, azide did.

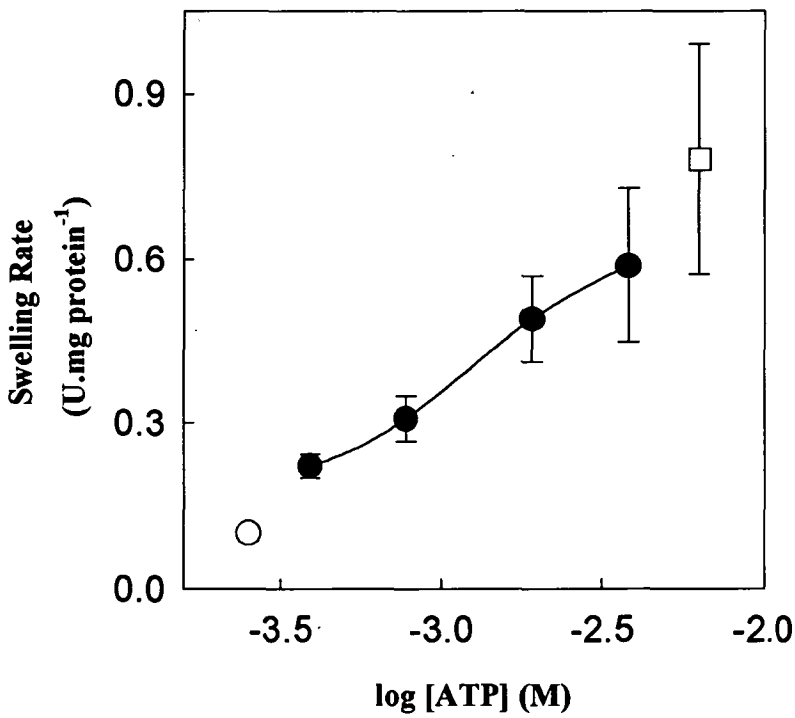


**Figure 3.7** Effect of ATP (●) on the swelling rate of skeletal muscle SS mitochondria in the presence of atractylate, oligomycin, Antimycin A and valinomycin. Basal swelling rate (O) and uncoupled or FCCP-induced (□) swelling rates are also shown. (n≥5). Values are means ± S.E.M. When not visible the error bars are within the symbol.

**Table 3.2** Subsarcolemmal skeletal muscle mitochondrial swelling rates. Effect of maximum physiological concentrations encountered during exercise on the ATP-induced swelling effect. The incubation media consisted of 0.1 M potassium acetate, 2 mM magnesium chloride, 12.1 μg/ml oligomycin, 7 μM atractyloside, 0.5 μg/ml antimycin A, 1.2 μM valinomycin and 10 mM Tris-maleate pH 7.4. Protein concentration was 0.2 $mg.ml^{-1}$. Swelling rates were measured at 526 nm at 37°C and are shown as means ± S.E.M. (n≥3). *, $P<0.02$ when compared with the mitochondria alone. †, $P<0.02$ when compared with the mitochondria plus 0.75 mM ATP.

| Order, and type of additions, after valinomycin | Swelling Rate ($U.mg\ protein^{-1}$) |
|---|---|
| | 0.100 ± 0.015 |
| 0.75 mM ADP | 0.094 ± 0.021 |
| 0.75 mM ATP | 0.308 ± 0.042* |
| 0.75 mM ADP + 0.75 mM ATP | 0.115 ± 0.042† |
| 1.5 mM Cr + 0.75 mM ATP | 0.248 ± 0.063* |
| 1.5 mM Pi + 0.75 mM ATP | 0.303 ± 0.097* |
| 1.5 mM CrP + 0.75 mM ATP | 0.244 ± 0.072* |
| 35 μM ADP + 1.5 mM (Pi + Cr) + 0.75 mM ATP | 0.276 ± 0.092* |

### 3.2.3.2 *Lack of ATP-induced* $\dot{V}O_2$

Although ATP was found to induce proton dependent swelling in SS skeletal muscle mitochondria, it did not induce mitochondrial oxygen consumption in the presence of atractylate or oligomycin. In the absence of these inhibitors, ATP was able to stimulate respiration in SS muscle mitochondria in the same as ADP (Fig3.8). In liver mitochondria, ATP did not stimulate respiration in either the presence or absence of the inhibitors. It was concluded that the ATP was being hydrolysed by non-mitochondrial ATPases present in the SS muscle mitochondria preparation and that either the prevention of ADP entry into the mitochondria (with atractylate) or its phosphorylation by the $F_0F_1$-ATPase (with oligomycin) effectively removed any stimulatory effect of the added ATP.

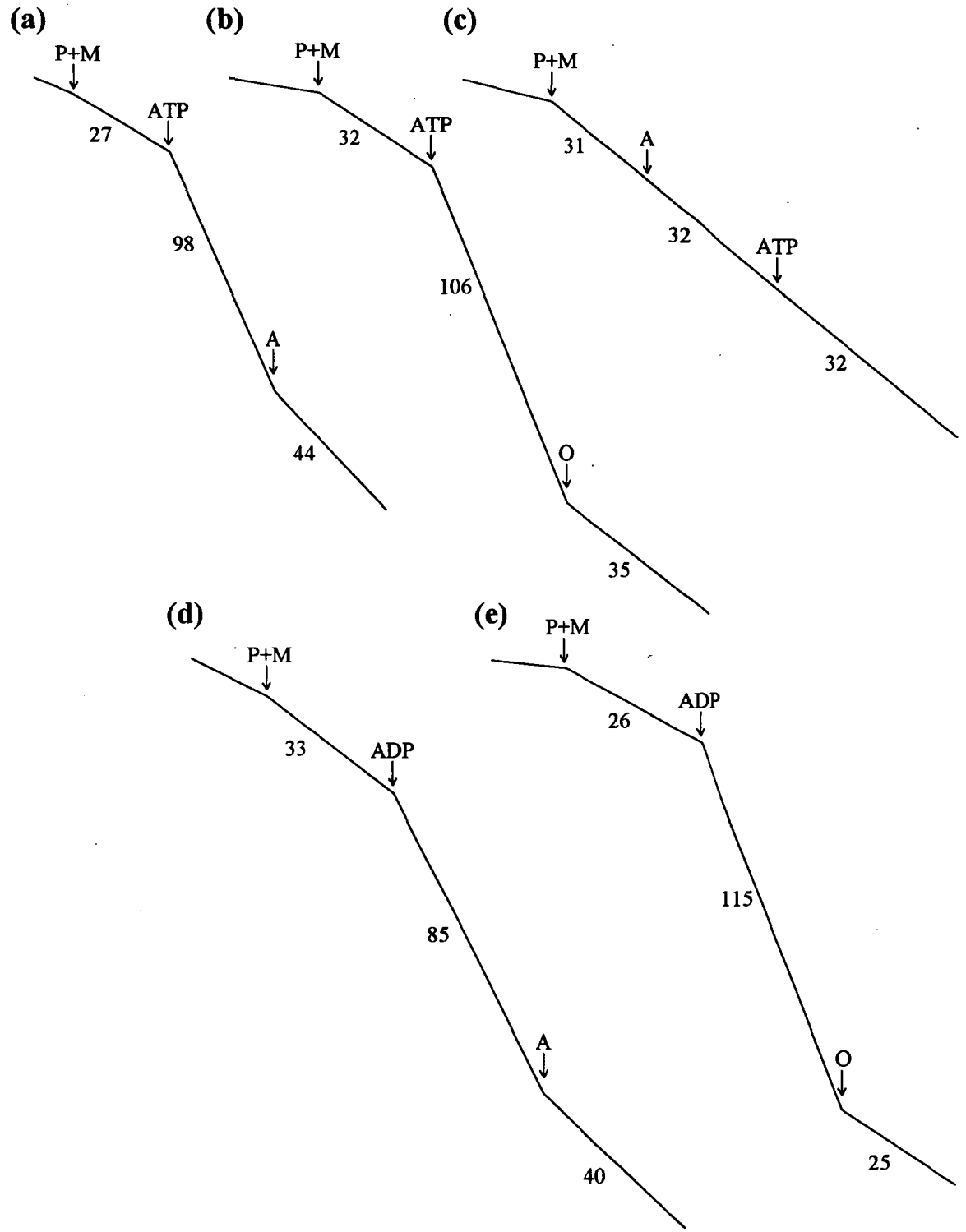


**Figure 3.8** Typical tracing of oxygen consumption by skeletal muscle mitochondria. (a) induction of respiration by ATP and inhibition by atractylate (A); (b) induction of respiration by ATP and inhibition by oligomycin (O); (c) failure of ATP to stimulate respiration in the presence of atractylate (A); (d) induction of respiration by ADP and inhibition by atractylate (A); (e) induction of respiration by ADP and inhibition by oligomycin (O). Figures represent rates of oxygen consumption in nmol $O.min^{-1}.mg\ protein^{-1}$.

### 3.2.4 *Discussion*

Swelling was able to be induced by the addition of ATP to SS skeletal muscle mitochondria. As this increase in proton permeability was similar to that seen by Prieto *et al.* (1992) in yeast mitochondria, the same conclusions were able to be drawn for this phenomenon in these muscle mitochondria. Due to the occurrence of this effect in the presence of atractyloside, oligomycin and Antimycin A, it might be surmised that:

(1) activation of the $K^+/H^+$ antiporter was not occurring, because in the absence of valinomycin, ATP did not induce swelling

(2) ATP-induced proton permeability was not dependent on either its presence in, or its translocation into, the matrix

(3) the proton translocation was not due to the $F_0F_1$-complex "running" backwards

and (4) addition of ATP was not forcing the electron transport chain backwards and hence importing rather than exporting the protons. This final point was supported by the finding that cyanide was unable to inhibit the ATP-induced rate of swelling. However, the examination of the inhibitory action of ATP analogues and azide yielded results suggesting that these suppositions (1 to 4, above) may not be entirely correct. Inhibition of the ATP-induced rate of swelling by ATP-γ-S and azide indicated that the observed effect may in fact have been due to the electron transport chain running in the reverse direction. This was further supported by the finding that AMP-PNP was also able to inhibit the induced rate of swelling. As this analogue is unable to be hydrolysed by the ATP-ases, movement of the electron transport chain in the reverse direction would also be inhibited.

This apparent contradictory data may just indicate that the site of egress of the protons is between Complexes II (cytochrome c reductase) and V (cytochrome c oxidase) where Antimycin A and cyanide, respectively, are thought to act on the electron transport chain. As azide, which inhibits the electron transport chain at Complex IV, does have an inhibitory effect this may suggest that this complex is involved in the swelling induced by the addition of ATP.

In a physiological system, if ATP is able to cause uncoupling in skeletal muscle, any recoupler is possibly something that changes (i.e. increases) with the onset of exercise so that muscle contraction is not compromised. During exercise, many changes occur in skeletal muscle. These include increases in the concentrations of creatine, phosphate and ADP as well as decreases in the concentration of creatine phosphate. As can be seen from Table 3.2, none of these obvious compounds were able to inhibit the ATP-induced swelling effect, except for ADP. The concentrations

of ADP required (equal to that of the added ATP - see Table 3.2) for this inhibition are higher than that seen in the cytosol in skeletal muscle even under maximal exercising conditions.

Due to the difficulties involved in gaining maximal ADP and minimal ATP concentrations in the mitochondrial matrix, the physiologically normal situation encountered when exercising could not be obtained. High matrix ADP and low ATP would be analogous to the external mitochondrial conditions needed in this assay for the inhibition of the ATP-induced swelling effect to be inhibited by ADP. As the assay requires the protons to leave the matrix, rather than to enter which is the normal physiological situation, the concentrations and effects of these two nucleotides on the external surface of the inner mitochondrial membrane are not unreasonable.

ATP possibly has a general effect on Complex IV, and is able to act from both the outer and inner surfaces of the inner mitochondrial membrane. This would account for the effect seen in this unphysiological swelling assay and the postulation of a similar effect, with the protons entering the mitochondrial matrix, in the physiological situation that occurs in vivo. Lack of a response to ATP in the respiration assays is possible due to contamination by other muscle ATPases that rapidly degrade ATP and generate sufficient ADP to cause inhibition of the stimulatory effect by any ATP that remains.

Due to the lack of any stimulatory effect in respiratory studies, the response seen in the swelling assays, which although similar to that reported for yeast by Prieto *et al.* (1992), is unlikely to be one that causes mitochondrial uncoupling in skeletal muscle *in vivo*. Thus, other uncoupling mechanisms were investigated.

## 3.3 *Substrates as potential uncouplers of skeletal muscle mitochondria*

### 3.3.1 *Introduction*

Mitochondria from skeletal muscle yield different respiratory control ratios (RCR = state III / state IV respiration), when given different substrates. Although all substrates including glutamate, pyruvate + malate, α-ketoglutarate and succinate are able to be oxidized at very good rates by these mitochondria (Table 3.3), the differences in the respiratory rate under State IV conditions indicates a possible uncoupling process that may be able to contribute to muscle thermogenesis.

**Table 3.3** Oxidative and energy-coupling activities of skeletal muscle mitochondria from rat hindlimb (Dow, 1967). $\dot{V}O_2$ is a measure of oxygen usage in microgram-atoms of Oxygen per mg of protein per hour. 100 mM glutamate; 100 mM pyruvate + 2.5 mM malate; 10 mM α-ketoglutarate + 10 mM malonate; 20 mM succinate + 3 mM amytal. Values are means ± S.E.M.

| Substrate | $\dot{V}O_2$, State IV | $\dot{V}O_2$, State III | RCR |
|---|---|---|---|
| Glutamate | 1.4 ± 0.9 | 43.6 ± 7.7 | 31.1 |
| Pyruvate+malate | 8.1 ± 2.4 | 40.6 ± 9.7 | 5.0 |
| α-Ketoglutarate+malonate | 1.5 ± 0.4 | 22.3 ± 2.8 | 14.9 |
| Succinate+amytal | 18.9 ± 5.1 | 30.9 ± 5.1 | 1.6 |

Skeletal muscle mitochondria have been shown to exhibit poor respiratory control when succinate is the added substrate, and as yet no explanation has been proposed for this phenomena. The rate of respiration induced on addition of succinate to the mitochondria is often unable to be further significantly stimulated by the addition of either ADP or an uncoupler. This action does not appear to be present in liver mitochondria where very good RCRs are obtained when succinate is the added substrate or with skeletal muscle mitochondria when supplied with substrates other than succinate.

To examine the possibility that a substrate could act as an uncoupler in skeletal muscle mitochondria, it was necessary to find a method of investigation that would allow differentiation between the coupled and uncoupled states despite small changes in respiratory activity.

### 3.3.1.1 *MTT*

MTT or [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] has been used by others to measure cell proliferation and the rate of activity of various respiratory enzymes (Mossman, 1983; Slater *et al.*, 1963). It is a water soluble salt which yields a yellow solution. The aqueous salt is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes. Production and mitochondrial accumulation of the formazan product occurs in proportion to the respiratory rate. The formazan product can be solubilized in isopropanol for spectrophotometric measurement that yields absorbance as a function of the concentration of the converted dye (catalogue Technical information). Originally our research group chose to use MTT in the perfused rat hindlimb in an attempt to identify the regions and cell types responsible for the NE-mediated increases in oxygen consumption. Surprisingly, it was found that the rate of conversion of MTT in the presence of NE (Fig. 3.9) was decreased when compared to the rate seen under basal conditions (S. Rattigan, K.A. Dora, A. Matthias, J. Newman, K.A. Miller, J.T. Steen, T.P.D. Eldershaw and M.G. Clark, unpublished observations 1995). In Section 3.3.3 it was found that a decrease in the MTT conversion rate occurred in uncoupled isolated mitochondria in conjunction with a decrease in membrane potential despite stimulated increases in the respiratory rate. Thus, the findings in the perfused hindlimb suggested that NE was creating an uncoupling of the skeletal muscle mitochondria.

### 3.3.1.2 *Membrane potential*

Proton pumping by the respiratory chain creates a proton gradient across the inner mitochondrial membrane which has two components - a membrane potential and a pH gradient. Both of these components store energy that can drive ATP synthesis. To measure the mitochondrial membrane potential, lipophilic cations and their uptake by the mitochondria have been used.

In hindlimb studies primarily designed to corroborate the above MTT findings, [$^3$H]-triphenylmethylphosphonium ([$^3$H]-TPMP) was used to measure the mitochondrial membrane potential (Fig. 3.10). It was found that significantly less was taken up by the different muscle groups when the vasoconstrictor-induced oxygen consumption occurred compared with basal, resting conditions. These data tended to suggest that some loss of membrane potential occurred when vasoconstrictors were used to induce increases in hindlimb oxygen consumption. Whether this involved only the mitochondrial membrane potential or included the cytosolic membrane potential was not clear.

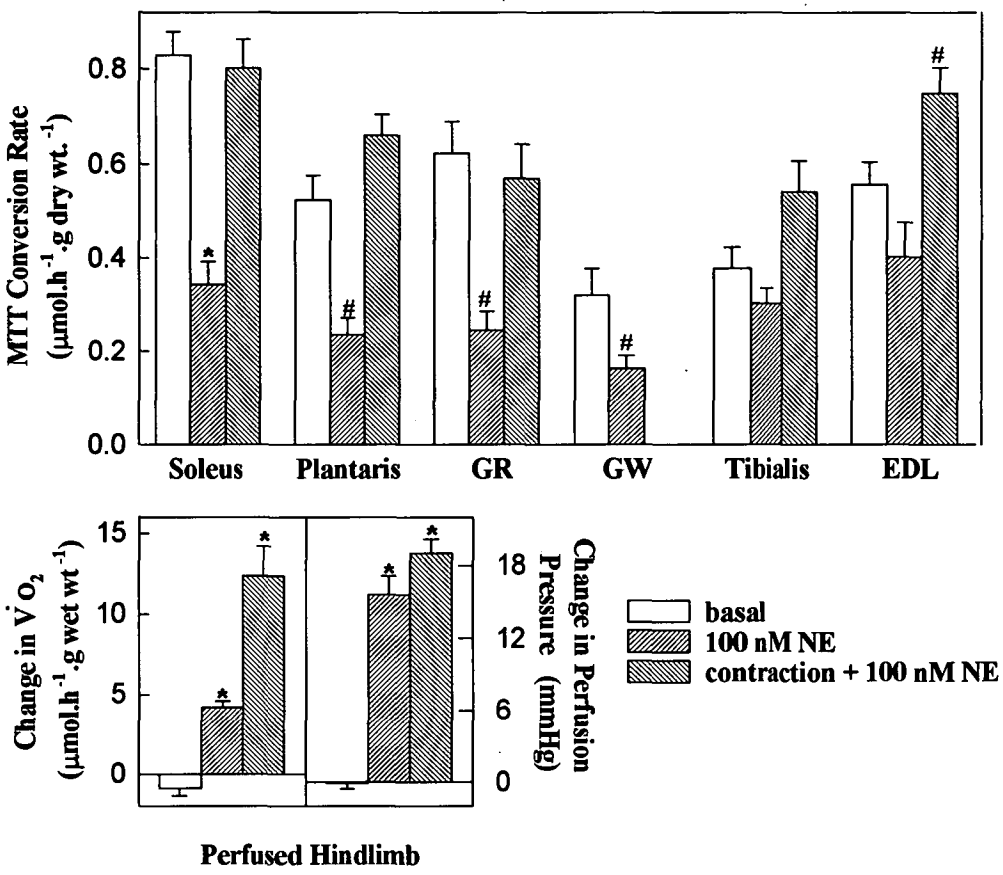


**Figure 3.9** MTT conversion rates by the constant flow perfused rat hindlimb under basal and NE conditions and during motor nerve stimulation with NE. After reaching steady state, MTT was infused for 30 minutes. Following a 10 minute washout period, the perfusion was terminated and the muscle groups excised, freeze dried and homogenized in 2 ml of isopropanolol. After centrifugation at 1000 g for 10 minutes, the absorbance of the supernatant was measured at 550 nm using a Milton Roy spectophotometer. The extinction coefficient of MTT was obtained using diaphorase plus NADH to completely convert the dye to the formazan product. GR and GW muscles were assayed together for contraction + 100 nM NE. GR = gastrocnemius red; GW = gastrocnemius white; EDL = extensor digitorum longus. (S. Rattigan, K.A. Dora, A. Matthias, J. Newman, K.A. Miller, J.T. Steen, T.P.D. Eldershaw and M.G. Clark, unpublished observations 1995). ($n > 3$). Values are means ± S.E.M. *, $P < 0.0001$ and #, $P < 0.05$ compared to basal conditions.

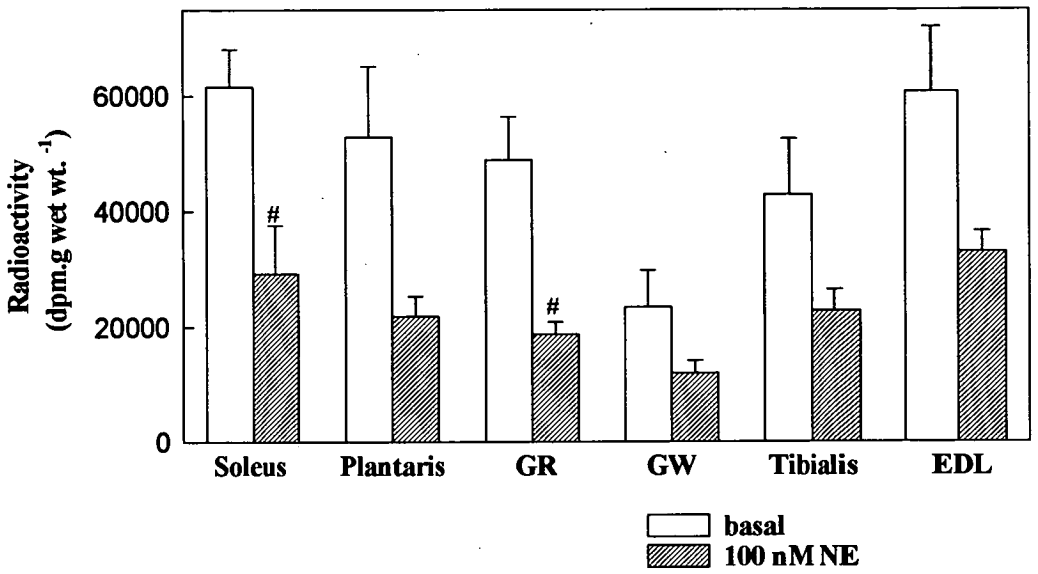


**Figure 3.10** Uptake of [$^3$H]-TPMP by muscles of the perfused rat hindlimb under both basal and NE-stimulated conditions. Oxygen and pressure are similar to that given in Fig. 3.9. Following a 15 min equilibration period (either basal or NE-infused), a solution containing [$^3$H]-TPMP and [$^{14}$C]-sucrose was infused for 15 min. Infusion was then stopped, and after a further 15 min the perfusion was halted and the muscles excised. The muscles were then homogenized in 2.5 g of distilled water, and the homogenate centrifuged for 15 min at 1700 g. The supernatant mass was measured and an aliquot was mixed with scintillation fluid and the radioactivity determined using a Beckman LS3801 liquid scintillation counter. Radioactivity was calculated as (dpm.g wet wt.$^{-1}$) according to the following calculation.

$$\frac{(^3\text{H}-(^{14}\text{C} \times \text{ratio})) \times \text{total supernatant volume} \times (\text{water wt.} + \text{muscle wt.})}{\text{vol. supernatant analysed} \times \text{muscle wt.} \times \text{supernatant wt.}}$$

where the ratio is $^3$H:$^{14}$C in the perfusate samples during infusion of the TPMP\sucrose solution. GR = gastrocnemius red; GW = gastrocnemius white; EDL = extensor digitorum longus. Values are means ± S.E.M. $^{\#}$, $P < 0.05$ when compared to basal conditions. (J. Newman, personal communication). (n=3).

As uncouplers are known to cause decreases in the membrane potential of mitochondria as well as increases in oxygen consumption, measurement of this parameter particularly at the level of isolated mitochondria was deemed necessary.

Rhodamine 123 (Rh123) is another membrane potential marker that has been used in studies of isolated mitochondria to assess their membrane potential and any changes that may be induced by varying conditions. It is a fluorescent, lipophilic cation dye that appears to distribute electrophoretically into the mitochondrial matrix in response to the membrane potential, not pH (Emaus *et al.*, 1986). Due to its low toxicity, this dye has been extensively used for the measurement of mitochondrial membrane potential in whole cells (reviewed by Chen, 1988), and it is the only fluorescent lipophilic cation whose uptake by mitochondria in living cells is completely inhibited in the presence of azide plus oligomycin.

In suspensions of isolated rat liver mitochondria, Emaus *et al.* (1986) found that a red shift of Rh123 absorbance and fluorescence occurred following mitochondrial energization. As the membrane potential increases, dye concentrations in the mitochondrial matrix increase with much of the internalized dye being bound. Concentration ratios (in-to-out) of Rh123 were found to approach 4000:1 (Emaus *et al.*, 1986). This fluorescent probe is a redistribution probe, as it redistributes across the mitochondrial membrane under the influence of the membrane potential.

### 3.3.2 *Methods*

Mitochondrial isolation, protein determination and respiration were as given in Section 3.2.2. Succinate (5 mM) + rotenone (5 μM) was also used as a substrate for these studies.

#### 3.3.2.1 *MTT*

The rate of conversion of MTT to the formazan product by mitochondrial suspensions was continuously measured using a Varian Cary 219 spectrophotometer at 600 nm. The formazan product has a maximal absorption at 550 nm, but that at 600 nm is still significant (Altman, 1973). This wavelength was chosen so as to avoid the maximal cytochrome absorption wavelengths. Cuvettes were air equilibrated and kept continuously stirring at 37°C. Basal mitochondrial rates were obtained prior to the addition of the MTT.

MTT conversion rates were calculated from the rate of change of absorbance, cuvette volume and protein content of the mitochondrial preparation.

### 3.3.2.2 *Rhodamine 123 membrane potential*

Rhodamine fluorescence by mitochondrial suspensions was continuously analysed using an Aminco-Bowman spectrofluorometer, with an excitation wavelength of 507 nm and an emission wavelength of 530 nm. Cuvettes were air equilibrated and kept continuously stirring at 37°C. Rhodamine was added to the cuvette prior to the addition of the mitochondria and substrate.

Membrane potential was not calculated. Instead, to allow comparisons between responses, all data have been expressed as a percentage change in fluorescence from the baseline level, using the state IV respiratory state as the standard 100%. The quantification of membrane potential in this manner has previously been used by Duchen and Biscoe (1992) when measuring the relative mitochondrial membrane potential of Type I cells isolated from the rabbit carotid body.

### 3.3.3 *Results*

Measurement of respiratory activity with different substrates (pyruvate + malate and succinate) in isolated SS skeletal muscle mitochondria showed differences depending on the respiratory state of the mitochondria (Figure 3.11). Membrane potential (Rh123 Fluoresence %) appeared identical for the two substrates for all the measured respiratory states although $\dot{V}O_2$ and MTT conversion rates indicated marked differences between them.

With pyruvate + malate as the added substrates, state IV respiration was low and the MTT conversion rate was high. On addition of ADP, little change was noted in the rate of conversion of the tetrazolium dye although the other measured parameters altered significantly with $\dot{V}O_2$ increasing and membrane potential decreasing. In the presence of an uncoupler $\dot{V}O_2$ could not be further increased and infact was slightly, though not significantly, reduced when compared to that obtained under state III respiratory conditions. This uncoupled $\dot{V}O_2$ was still above that found under state IV conditions. Conversion of MTT was markedly inhibited in the presence of an uncoupler and membrane potential was decreased to the levels encountered before the addition of substrate to the mitochondria.

When succinate was present as the substrate, the state III and uncoupled respiratory states showed similar $\dot{V}O_2$, MTT conversion rates and membrane potentials to those seen with pyruvate + malate as the substrate. However, differences were apparent under state IV respiratory conditions. Thus, unlike the results gained for pyruvate + malate where respiration was low and the MTT

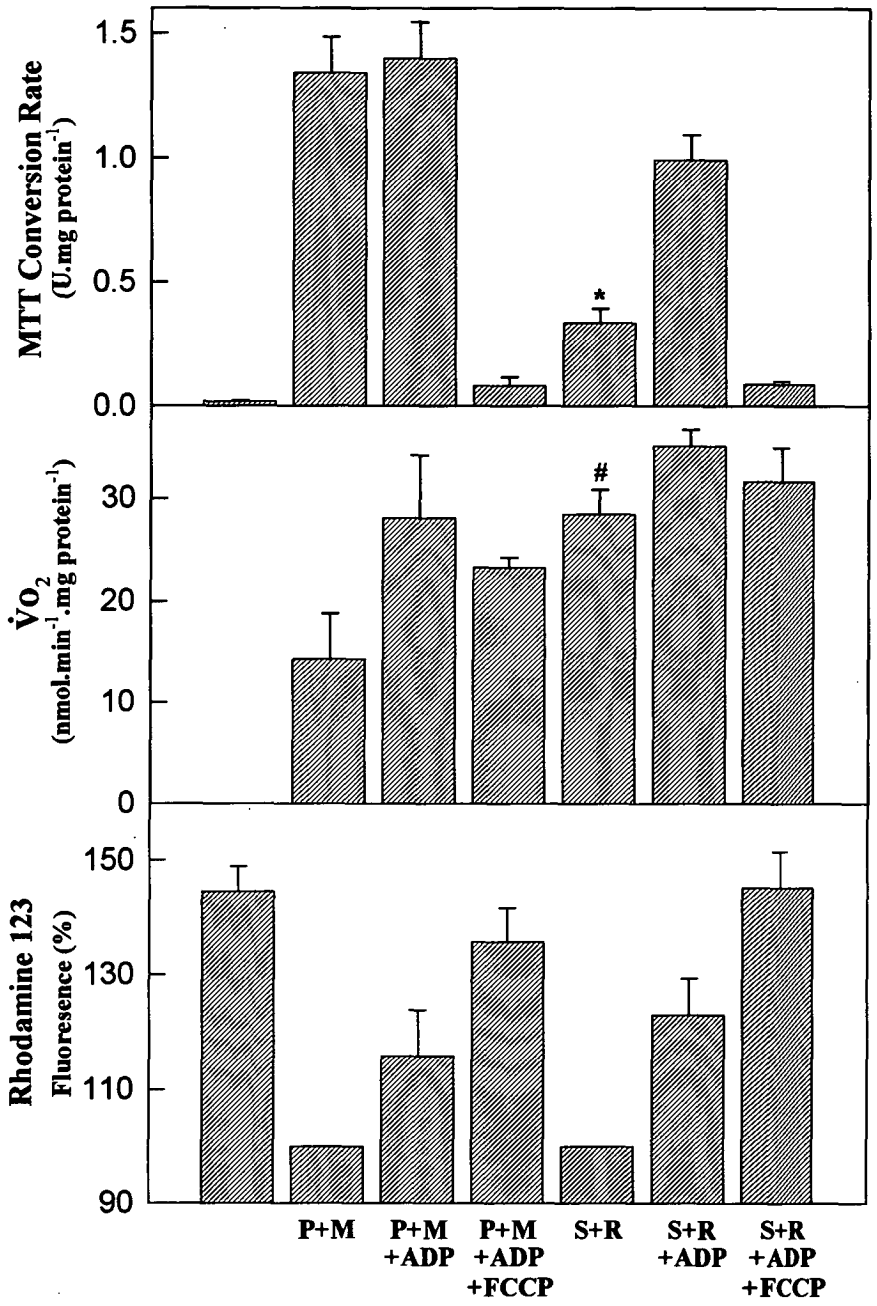


**Figure 3.11** Comparison of MTT conversion rates, respiration and membrane potential of SS skeletal muscle mitochondria in the presence of two different substrate combinations. Mitochondria alone, state IV (substrate), state III (substrate + 200 μM ADP) and uncoupled (substrate + 200 μM ADP + 6 μM FCCP) conditions have been measured at 37°C. P+M = 5 mM pyruvate + 5 mM malate; S+R = 5 mM succinate + 5 μM rotenone. (n=3-4). Values are means ± S.E.M. *, $P < 0.001$ and #, $P < 0.05$ when compared to pyruvate + malate substrate conditions.

conversion rate was high, state IV $\dot{V}O_2$ with succinate was already high and the rate of conversion of MTT was markedly depressed.

The addition of ADP to the respiratory medium was able to overcome the inhibition of the MTT conversion rate evident when succinate was the substrate. ADP increased the MTT conversion rate from 0.334 ± 0.057 to 0.990 ± 0.103 U. mg protein$^{-1}$ when succinate was the substrate. Furthermore, atractylate and oligomycin were each able to inhibit this effect back to a rate similar to that found in the absence of ADP. MTT conversion rates in the presence of oligomycin or atractylate and ADP were 0.273 ± 0.010 and 0.222 ± 0.012 U. mg protein$^{-1}$, respectively.

Unlike SS skeletal muscle mitochondria, the pattern of results gained with liver mitochondria for both of the examined substrate combinations (succinate and pyruvate + malate) were similar (data not shown). This pattern with liver mitochondria was the same as that found for SS skeletal muscle with pyruvate + malate as the substrate. On addition of ADP, the respiratory rate increased, membrane potential decreased and the MTT conversion rate remained unchanged. With the subsequent addition of FCCP, the respiratory rate was further increased and both membrane potential and the MTT conversion rate were returned to the rates seen prior to substrate addition.

### 3.3.4 *Discussion - Succinate involvement in skeletal muscle thermogenesis*

Skeletal muscle mitochondria possess a reaction to succinate that is not present in liver mitochondria. With respect to both $\dot{V}O_2$ and the MTT conversion rates, this substrate acts in a manner similar to that seen in either liver or SS skeletal muscle mitochondria on the addition of a known uncoupler such as FCCP or PCP. In a physiological system, this substrate may be able to act as an uncoupler specific to muscle mitochondria.

In keeping with a physiological uncoupling role for succinate, recoupling is readily achieved by the transition of the mitochondria from state IV to state III respiration. Results gained when either ADP entry into the mitochondrial matrix (with atractylate) or ADP phosphorylation (with oligomycin) were prevented indicate that the presence of the diphosphate within the mitochondrial matrix was not enough to overcome the succinate inhibitory effect and that, infact, the active phosphorylation of ADP was necessary before maximal MTT conversion rates were attained.

An alternate explanation for this data is that the preparation is contaminated with membrane fragments that are able to oxidize succinate and thus stimulate the apparent respiartory rate. These fragments could thus be responsible for the increased rate of oxygen usage under state IV conditions but have no influence on the

measurements of membrane potential. This explanation for the data is unlikely as firstly, there is no NADH-induced respiratory rate which is the usual method of examination for mitochondrial fragments; secondly, as it is succinate dehydrogenase which is thought to be responsible for the conversion of MTT the increased respiratory rate should be reflected as an increase and not a decrease in the rate of conversion of the tetrazolium dye; and thidly, the activity of the electron transport chain should already be maximal in fragments and not further stimulated by the addition of either ADP or FCCP. As the MTT conversion rate was shown to be increased on addition of ADP and decreased again with the subsequent addition of FCCP, the data suggests that it is intact mitochondria and not fragments that are responsible for the high respiratory rate that is found.

Although succinate appears to possess $\dot{V}O_2$-stimulatory and MTT-inhibitory actions in SS skeletal muscle mitochondria, it is probably not responsible for the conditions encountered in the perfused rat hindlimb on addition of norepinephrine (Fig. 3.9). This is indicated by differences in membrane potential ([$^3$H]-TPMP) data between the perfused hindlimb, where it is decreased on addition of norepinephrine (Fig. 3.10), and the isolated mitochondria where it is unchanged (Fig. 3.11) despite the stimulation of $\dot{V}O_2$.

The examination of the effect of succinate on mitochondria from another species, and a similar comparison to the effect of pyruvate + malate, may further clarify the situation as to whether succinate does have a general interspecies role in muscle thermogenesis.

## 3.4 *Comparative study with duckling skeletal muscle mitochondria*

### 3.4.1 *Introduction*

If another major mechanism for thermogenesis, similar to that in BAT exists, then it is possibly common to most homeothermic species - i.e. eutherians, marsupials and birds. The results gained in Section 3.3 suggest that succinate may be able to act as a physiological uncoupler of skeletal muscle mitochondria. This substrate appears to be able to stimulate the maximal respiratory activity of these mitochondria without affecting the membrane potential. Despite this apparent uncoupling action, ATP levels are easily maintained as ADP appears to reverse the uncoupling action, leaving succinate free to then act solely as the substrate.

To further assess the possibility of succinate as a widespread physiological uncoupler, these three parameters (respiration, membrane potential and MTT conversion rates) were measured in a species that does not possess BAT. The IMF and SS skeletal muscle mitochondrial populations from the duckling red gastrocnemius muscle were isolated and examined for the presence of this succinate-induced uncoupling action. As any thermogenic mechanism that may be present in skeletal muscle should be upregulated with adaptation to cold, both mitochondrial subpopulations for both thermoneutral (TN) and cold acclimated (CA) ducklings were examined.

### 3.4.2 *Methods*

These experiments were conducted in the Laboratoire de Thermorégulation et Energétique de l'Exercise at the Universite Claude Bernard Lyon, Lyon, France during a three week visit in October 1994.

#### 3.4.2.1 *Duckling subsarcolemmal and intermyofibrillar mitochondria isolation*

Essentially, the method employed for the isolation of the duckling muscle mitochondria was similar to that used for the rat skeletal muscle mitochondria. Ducklings were housed in groups at either 25°C for the first week and then half were transferred to 4°C for the next 4 weeks. After decapitation, the gastrocnemius muscles were removed from both hindlimbs, and the red section of the muscle was removed, cleaned of extraneous tissue, weighed, minced and homogenized in isolation buffer (100 mM sucrose, 50 mM KCl, 5 mM EGTA and 50 mM Tris base (pH 7.4) using a Potter-Elvehjem homogenizer (10 ml per g of muscle). The homogenate was then centrifuged at 800 g for 10 minutes, and the supernatant decanted and stored.

The pellet was resuspended in 40 ml of isolation buffer and incubated for 5 minutes on ice with 1 mg Nagarase per gram of tissue. This was diluted with a further 40 ml of isolation media prior to homogenization (5 passes with the Potter-Elvehjem homogenizer). Both the supernatant and digested pellet were then centrifuged at 1000 g for 10 minutes. Keeping the two mitochondrial populations separate, the supernatants were recentrifuged at 8700 g for a further 10 minutes. The resulting pellets were washed twice in 10 ml of isolation media and recentrifuged at 8700 g. The final pellets were resuspended in storage media (250 mM sucrose, 1 mM EGTA and 20 mM Tris base pH 7.4 with KCl).

The mitochondria isolated from the original supernatant were the SS, and those from the Nagarase digested pellet were the IMF.

### 3.4.2.2 *Protein determination - Biuret method*

Duckling mitochondrial protein content was determined using the Biuret method. Briefly, 20 µl of the mitochondrial suspension was mixed with 0.98 ml distilled water, 0.5 ml 3% deoxycholic acid and 1.5 ml Biuret reagent. The mixture was then boiled for 1.5 minutes, cooled, and the absorbance measured at 540 nm. BSA was again used as the protein standard.

### 3.4.2.3 *Assays*

Methods for determination of MTT conversion rates and mitochondrial membrane potential (using Rh123) were essentially the same as given in Section 3.3.2, except that the respiration buffer and some final concentrations of additions differed slightly. All assays were performed at 25°C.

The basic respiration buffer was: 200 mM sucrose, 5 mM $KH_2PO_4$, 0.2% fatty acid free bovine serum albumin and 20 mM Tris-HCl pH 7.4 with KOH. Final concentrations for other additions were: substrates (succinate, pyruvate and malate) 5 mM, rotenone 100 µM, FCCP 4 µM, ADP 100 µM, MTT 22 µM and Rh123 65 nM. In each assay, the mitochondrial concentration was 0.5 mg/ml for the respiration and Rhodamine 123 experiments, and 0.25 mg/ml for the MTT experiments. Respiration rates were measured using a Clark type oxygen electrode, a Kontron Uvikon 860 spectrophotometer for MTT conversion rates and a Perkin-Elmer MPF-3 fluorescent spectrophotometer with a Hitachi recorder for membrane potentials.

Calculations were as given previously. All results are expressed as mean ± S.E.M.

### 3.4.3 *Results*

Duckling skeletal muscle mitochondria, SS and IMF, were found to respond to both of the examined substrates in a similar manner.

$\dot{V}O_2$ was increased from state IV on the addition of ADP for both subsets of mitochondria, for the two acclimation temperatures and with both substrates. Absolute rates of respiration, both state IV and state III, were greater with succinate as the substrate and with the IMF mitochondria (Table 3.4). Of interest in the respiratory studies was the differing reaction to the addition of an uncoupler (FCCP). TN IMF mitochondria increased their $\dot{V}O_2$ above the state III rate using either substrate, but CA IMF mitochondria only did this with succinate (Fig.'s 3.11 and 3.12). In the presence of pyruvate + malate, these CA IMF mitochondria showed an uncoupled respiratory rate below that seen in state III. The SS mitochondria also showed this same respiratory decrease (state III compared to uncoupled) with both substrates and both acclimation temperatures.

The MTT conversion rate varied depending on the substrate present, the acclimation temperature of the ducklings and the population of mitochondria (Table 3.4). The mitochondria alone (i.e. with no added substrate) converted the tetrazolium dye at very slow rates (0.045±0.009 for TN IMF, 0.049±0.008 for CA IMF, 0.031±0.009 for TN SS and 0.026±0.003 U.mg protein$^{-1}$ for SS CA) and similar rates were also seen on the addition of an uncoupler. Although TN IMF mitochondria already converted the dye at high rates under state IV conditions with either substrate, CA further increased the rate (Fig. 3.12). State III conversion rates with pyruvate + malate were similar to those obtained under state IV conditions, but with succinate, state III rates were significantly reduced. Conversion rates of the SS mitochondria were well below those obtained for the IMF mitochondria (0.445±0.037 for TN IMF compared to 0.110±0.021 for TN SS) but the state III decrease with succinate and the lack of change with pyruvate + malate (Fig. 3.13) was still present.

The decrease in membrane potential (or the increase in Rh123 fluoresence) seen on the transition of the mitochondria from state IV to state III differed little from one population of mitochondria to the other, and with CA (Table 3.4). However, significant differences between the two populations were noted when the mitochondria were uncoupled.

Despite the variations present between the two substrates for all parameters, the change in acclimation temperature of the ducklings from 25°C to 4°C had no major effect on the skeletal muscle mitochondria under either state III or uncoupled respiratory conditions. This is shown when all three parameters are graphed against

**Table 3.4** Duckling skeletal muscle mitochondria - respiration, MTT conversion rates and membrane potential - for thermoneutral (TN) and cold acclimated (CA) ducklings. Two mitochondrial populations, the intermyofibrillar (IMF) and the subsarcolemmal (SS), were isolated from the red gastrocnemius muscle for comparison with the two substrates. *, $P < 0.05$ for CA compared with TN under the same conditions. All values are means ± S.E.M. ($n > 3$).

| | | succinate + rotenone | | | pyruvate + malate | | |
|---|---|---|---|---|---|---|---|
| | | state IV | state III | FCCP | state IV | state III | FCCP |
| Oxygen uptake | IMF-TN | 22.01±1.17 | 112.78±9.7 | 129.90±12.05 | 4.89±0.99 | 50.40±7.55 | 71.28±8.23 |
| (natoms.min$^{-1}$. | IMF-CA | 19.58±0.54 | 116.66±3.57 | 127.57±3.78 | 7.96±1.08 | 77.75±7.95 | 65.25±8.55 |
| mg protein$^{-1}$) | SS-TN | 9.96±1.08 | 26.45±2.17 | 20.64±2.24 | 2.25±0.85 | 24.12±4.54 | 14.03±1.36 |
| | SS-CA | 10.02±1.04 | 32.37±2.17 | 21.58±0.94 | 8.55±1.23* | 27.34±3.07 | 19.31±1.91 |
| MTT Conversion | IMF-TN | 0.445±0.037 | 0.202±0.020 | 0.024±0.006 | 0.527±0.016 | 0.591±0.050 | 0.028±0.013 |
| Rate | IMF-CA | 0.624±0.094 | 0.244±0.062 | 0.046±0.010 | 0.663±0.046 | 0.635±0.074* | 0.035±0.012 |
| (U.mg protein$^{-1}$) | SS-TN | 0.110±0.021 | 0.079±0.018 | 0.023±0.003 | 0.344±0.053 | 0.286±0.057 | 0.049±0.019 |
| | SS-CA | 0.179±0.021 | 0.128±0.028 | 0.024±0.010 | 0.274±0.018 | 0.302±0.022 | 0.018±0.003 |
| | IMF-TN | 100 | 117±1 | 192±2 | 100 | 114±4 | 173±2 |
| Rhodamine123 | IMF-CA | 100 | 115±1 | 190±2 | 100 | 117±0 | 172±1 |
| Fluoresence (%) | SS-TN | 100 | 121±5 | 177±5 | 100 | 112±3 | 153±9 |
| | SS-CA | 100 | 120±1 | 179±3 | 100 | 118±1 | 160±2 |

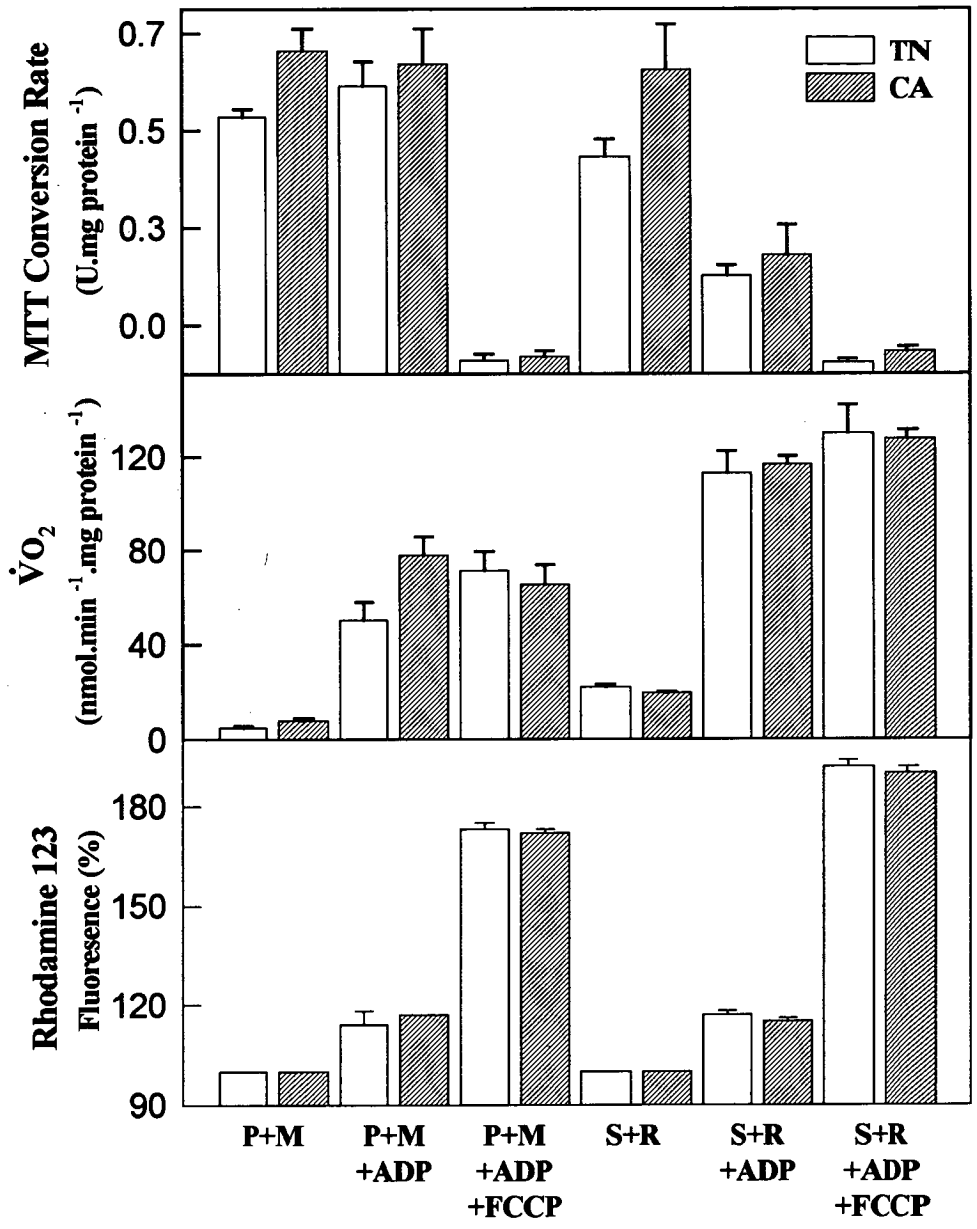


**Figure 3.12** Comparison of MTT conversion rates, respiration and membrane potential of IMF skeletal muscle mitochondria from either thermoneutral (TN) or cold acclimated (CA) ducklings using two different substrate combinations. All assays used respiration media (Section 3.4.2.3). State IV, state III (substrate + 200 μM ADP) and uncoupled (substrate + 200 μM ADP + 6 μM FCCP) conditions have been measured at 37°C. P+M = 5 mM pyruvate + 5 mM malate; S+R = 5 mM succinate + 100 μM rotenone. (n=3).

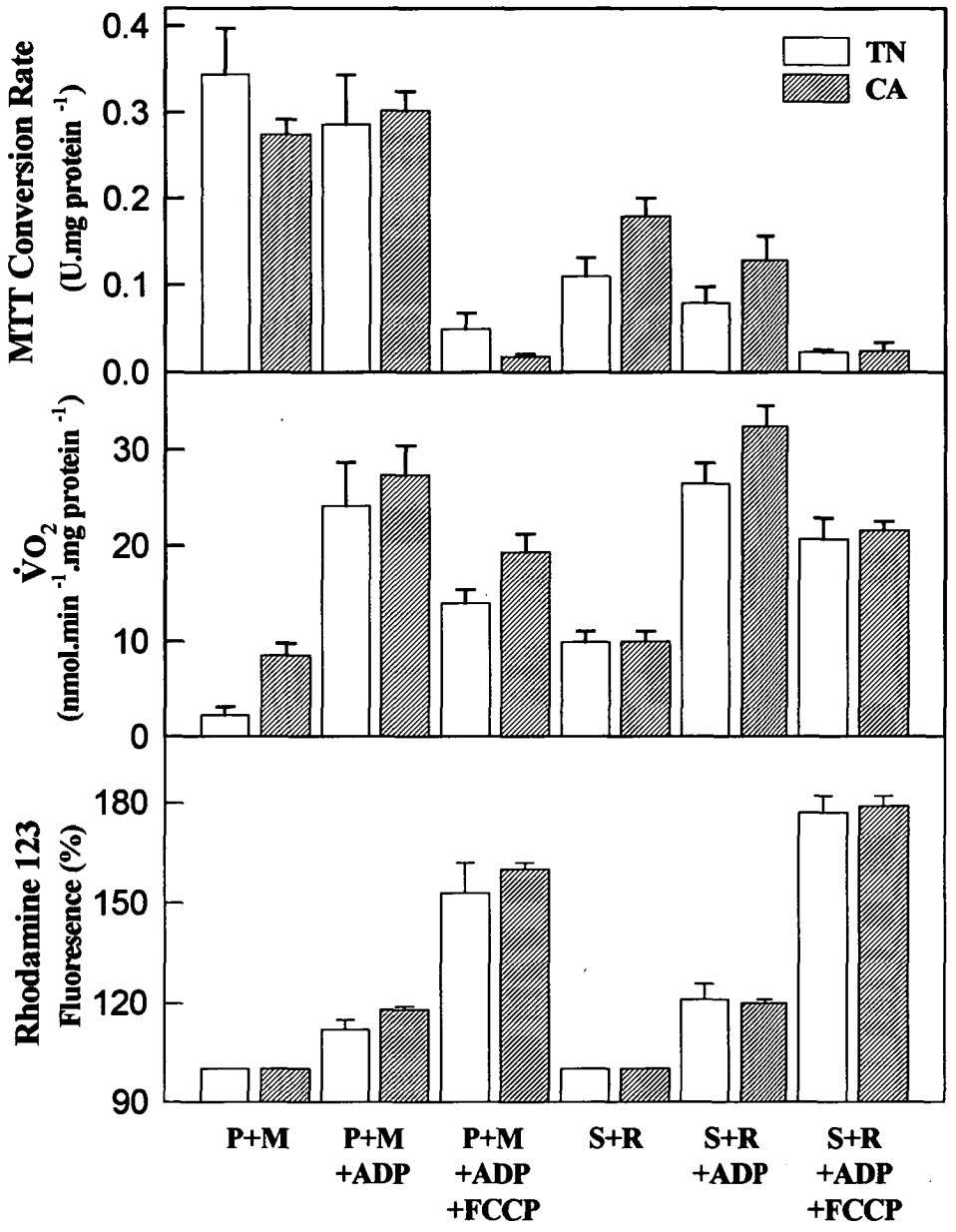


**Figure 3.13** Comparison of MTT conversion rates, respiration and membrane potential of SS skeletal muscle mitochondria from either thermoneutral (TN) or cold acclimated (CA) ducklings using two different substrate combinations. All assays used respiration media (Section 3.4.2.3). State IV, state III (substrate + 200 μM ADP) and uncoupled (substrate + 200 μM ADP + 6 μM FCCP) conditions have been measured at 37°C. P+M = 5 mM pyruvate + 5 mM malate; S+R = 5 mM succinate + 100 μM rotenone. (n=3).

each other (Fig. 3.14) for both sets of mitochondria from both TN and CA ducklings. State IV conditions, however, were found to vary, especially for the SS population.

The mitochondrial membrane potential, in the absence of substrate, was found to be lower than that gained in the presence of rotenone (Table 3.5), possibly indicating some residual electron flow through complex I of the respiratory chain. A high concentration of rotenone (100 μM) was used in the assays involving succinate so that this electron movement through complex I of the respiratory chain was inhibited. The membrane potential of the non-respiring (no added substrate) mitochondria observed in the presence of this 100 μM concentration of rotenone was thus equivalent to that obtained in the presence of both substrate and uncoupler. State IV $\dot{V}O_2$ was unaffected by this high concentration of rotenone, but both state III and uncoupled $\dot{V}O_2$ were significantly reduced due to the removal of this apparent complex I component (data not shown).

**Table 3.5** Duckling mitochondrial membrane potential differences due to the addition of rotenone. State IV respiration is taken as the reference (100%) position. Mitochondria, intermyofibrillar (IMF) or subsarcolemmal (SS), were isolated from the gastrocnemius red muscle of either thermoneutral (TN) or cold acclimated (CA) ducklings. All assays used respiration media (Section 3.4.2.3). Values are means ± S.E.M. (n=3).

| Mitochondria | no rotenone | 100 μM rotenone |
|---|---|---|
| IMF TN | 113±3% | 187±1% |
| IMF CA | 115±0% | 183±3% |
| SS TN | 113±2% | 172±3% |
| SS CA | 115±1% | 174±1% |

### 3.4.4 *Discussion*

The stimulation of $\dot{V}O_2$ and the inhibition of the rate of conversion of MTT seen with succinate as the substrate in rat SS skeletal muscle mitochondria (Section 3.3) was not found to be present in the SS or IMF mitochondrial populations isolated from the red gastrocnemius muscles of ducklings reared at either 25°C or 4°C. These data, together with the lack of variation in membrane potential deemed necessary for the stimulator of skeletal muscle thermogenesis (Fig. 3.10) in the perfused hindlimb, suggest that succinate is not able to act as a widespread physiological uncoupler. If

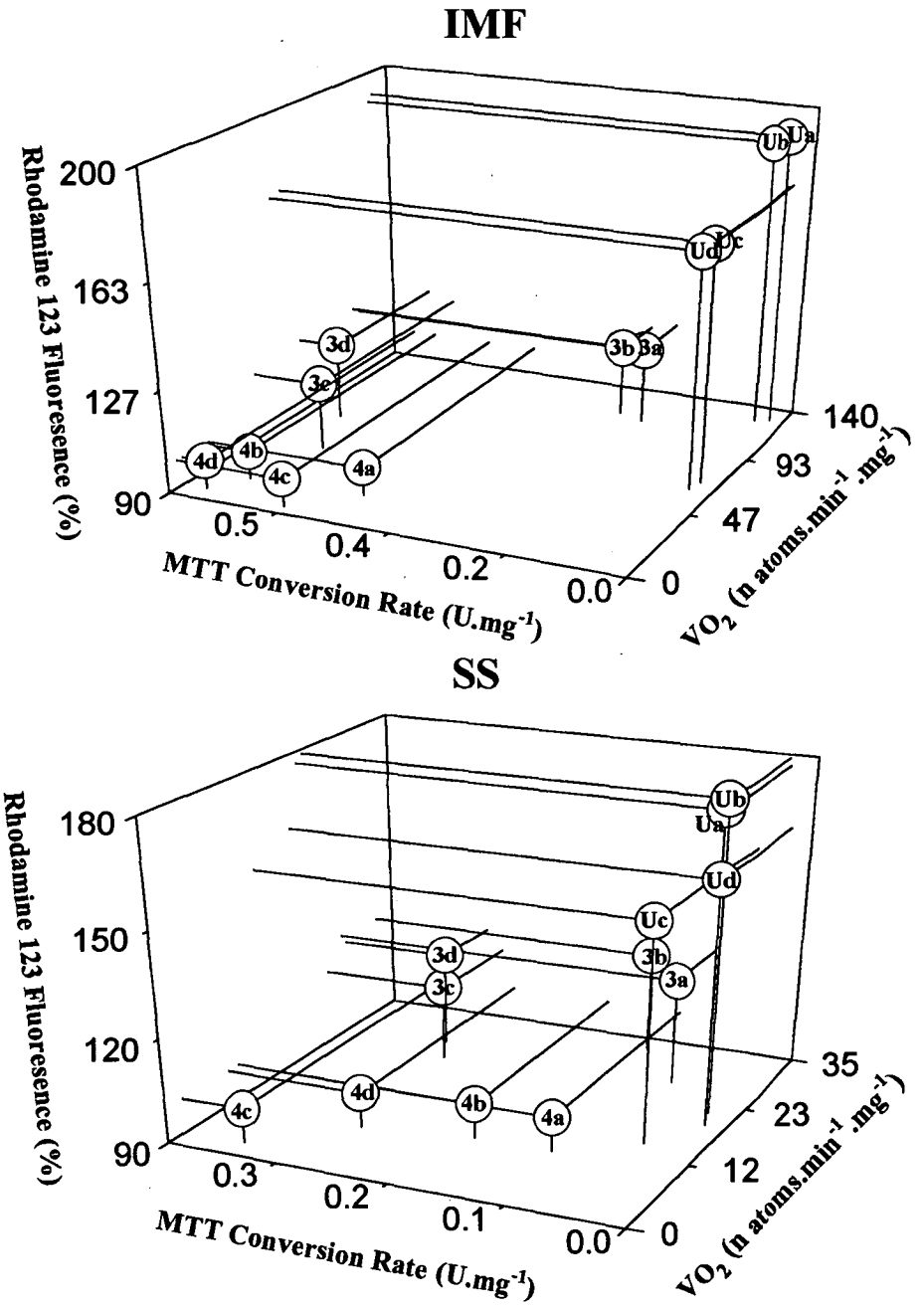


**Figure 3.14** 3-D graph of $\dot{V}O_2$ vs MTT conversion rate vs Rh123 membrane potential for the SS and IMF mitochondria isolated from duckling skeletal muscle (Section 3.4.2.1) from animals raised at either 25°C (thermoneutrality or TN) or 4°C (cold acclimated or CA). The lines are drop lines to each of the axes to orient the points in space. TN + succinate (a); CA + succinate (b); TN + pyruvate + malate (c); CA + pyruvate + malate (d). All substrate concentrations were 5 mM, and all assays were done using respiration media (Section 3.4.2.3). 4 = State IV (substrate); 3 = State III (substrate + ADP); U = uncoupled respiratory states (substrate + ADP + FCCP). (n=3).

succinate does have an uncoupling action then that action is possibly restricted to the rat, where expression of the uncoupling protein of BAT also occurs.

### 3.4.4.1 *Effects of cold acclimation*

Cold acclimation was found to influence SS mitochondria from skeletal muscle in two ways. Firstly, they showed a lower maximal respiratory rate (state III and uncoupled) and secondly, state IV respiration, with pyruvate + malate as the substrate, was increased markedly (Fig. 3.13). IMF mitochondria did not exhibit this same marked increase in state IV $\dot{V}O_2$ (Fig. 3.12). Figure 3.15, where all measured parameters ($\dot{V}O_2$, MTT conversion rate and membrane potential) have been graphed against each other, not only shows this alteration of state IV respiration with cold acclimation but also clearly illustrates that the state III and uncoupled respiratory states are not affected to the same extent. In addition, the SS mitochondria exhibit greater changes in response to cold acclimation than the IMF. Furthermore it is also evident that the applied substrate can have a major effect on the overall state of the mitochondria under all respiratory conditions. This is further evidence that it is this component of mitochondrial respiration (basal or maintenance level) that is involved with thermogenesis, at least in ducklings and that one population of mitochondria, the SS, from skeletal muscle may have a greater role in thermogenesis than the other.

During cold exposure, an increase in total leg muscle blood flow has been demonstrated in both TN and CA ducklings (Duchamp and Barre, 1993). In CA ducklings, increases in muscle fibre capillary density and the oxidative capacity of the skeletal muscle as well as looser coupling of the isolated mitochondria have been observed (Duchamp *et al.*, 1992). Glucagon treatment is able to mimic many of these cold-induced changes in the duckling skeletal muscle and mitochondria (Duchamp *et al.*, 1992) and when injected *in vivo*, glucagon also stimulates thermogenesis (Barre *et al.*, 1987a) and oxygen uptake (Duchamp *et al.*, 1993) in both TN and CA animals. Glucagon is thought to cause uncoupling of the mitochondria through the release of fatty acids (Barre *et al.*, 1989) which have been shown to increase the respiratory rate of isolated duckling mitochondria from both skeletal muscle and liver (Barre *et al.*, 1986). This response, to fatty acids, is enhanced in both types of mitochondria from cold acclimated animals.

Differences between bird and mammal, or duckling and rat, in the changes exhibited by muscles in response to cold exposure have been examined. The oxidative capacity of duckling skeletal muscle increased in response to cold exposure (+33% in gastrocnemius and +195% in pectoral) whereas that of the rat decreased (-22% in gastrocnemius) with cold acclimation (Barre *et al.*, 1987b). Opposite changes in the oxidative activity of rat skeletal muscle, specifically the gastrocnemius muscle, in

response to cold acclimation have been reported; -22% (Barre *et al.*, 1987b) and +32% (Hannon, 1960). In ducklings, these increases in oxidative capacity have been further classified for both the SS (+96% in gastrocnemius and +58% in pectoralis) and IMF (+51% in gastrocnemius and +33% in pectoralis) mitochondrial fractions (Goglia *et al.*, 1993). With variations such as these present in the literature in response to cold acclimation (between species as well as within a species), it is difficult to conclusively state whether these two species do or do not have a similar response to cold, and hence a similar mechanism for heat production.

### 3.4.5 *Conclusion*

The results of these studies lessened the possibility that succinate might serve as a general signal substance and substrate for skeletal muscle mitochondrial thermogenesis. Thus the following section explores the possibility that fatty acids fulfil this role.

## 3.5 *Fatty acids as mediators of skeletal muscle thermogenesis*

### 3.5.1 *Introduction*

Together with the changes in perfusion pressure and $\dot{V}O_2$ induced by the infusion of norepinephrine (Figs. 3.1 and 3.2), increases in the effluent concentration of glycerol have also been reported for both rat (Clark *et al.*, 1995; Clark *et al.*, 1994) and bettong (Ye *et al.*, 1995) perfused hindlimb. This enhanced release of glycerol suggested the presence of increased levels of fatty acids, and hence the possibility that the induced increase in $\dot{V}O_2$ could be due to uncoupling by fatty acids.

BAT thermogenesis is substrate-mediated, with fatty acids acting as both the stimulus and the fuel for the uncoupling of the mitochondria (Section 1.3.3). Free fatty acids have been shown to cause increases in $\dot{V}O_2$ in mitochondria isolated from rat liver (Pressman and Lardy, 1956; Nobes *et al.*, 1990a; Rottenberg and Hashimoto, 1986; Luvisetto *et al.*, 1990; Andreyev *et al.*, 1989; Mokhova *et al.*, 1993; Starkov *et al.*, 1994), rat skeletal muscle (Andreyev *et al.*, 1989; Mokhova *et al.*, 1993; Starkov *et al.*, 1994), duckling skeletal muscle (Barre *et al.*, 1986) and even pea stem (Petrussa *et al.*, 1992). In these tissues, although the fatty acids appear able to stimulate $\dot{V}O_2$, they do so without neccessarily acting as the fuel.

With the differences between the tissues, it is possible that skeletal muscle may either possess a protein similar to the uncoupling protein of BAT (Section 3.2.1.1) or an analogous thermogenic mechanism that also involves fatty acids. Mitochondrial $\dot{V}O_2$, membrane potential and MTT conversion rates were thus assessed in the presence of palmitate to see if they met the criteria of a putative uncoupler that could account for the increase in $\dot{V}O_2$ observed in the perfused rat hindlimb on addition of NE.

### 3.5.2 *Methods*

Mitochondria were isolated as described in Sections 3.2.2 and assays were as given in Section 3.3.2. Only 5 mM pyruvate + 5 mM malate was used as the substrate in these assays.

A palmitate-albumin suspension was prepared by mixing 92.3 mg palmitic acid (Mr 256.4) with 350 µl of 1 M NaOH and 7 ml of distilled water. After heating for 30 minutes at 75-90°C, 10 ml of water was added and the heating continued for a further 15 min. The solution was allowed to cool to 50-60°C and then mixed with 20 ml of water containing 5% BSA. The final preparation was 10 mM palmitate in 2.7% BSA.

### 3.5.3 *Results*

On the addition of 0.4 mM palmitate to respiring mitochondria, $\dot{V}O_2$ was increased by 7.80 ± 1.29 nmol.min$^{-1}$.mg protein$^{-1}$ (a significant increase of 42% above basal using the paired 2-tailed Student's *t*-test). Together with this increased respiratory rate, both MTT conversion rates and membrane potential were decreased in a dose dependent manner (Fig. 3.15). The MTT conversion rate dropped from 2.130 ± 0.309 to 0.161 ± 0.023 U. mg protein$^{-1}$ ($P < 0.003$) and the Rh123 fluoresence increased to 146 ± 2% ($P < 0.0001$) of the state IV membrane potential level.

If fatty acids were added to the mitochondria in the absence of any other substrate, no increases in $\dot{V}O_2$ were seen and no change in membrane potential or MTT conversion rates occurred.

Liver mitochondria displayed similar results for all three parameters on addition of methyl-β-cyclodextrin encapsulated oleate (2 mM) to the respiratory medium. Even allowing for considerable binding of the oleate to the albumin present in the respiration media, $\dot{V}O_2$ was increased by 25.3 ± 3.6 nmol.min$^{-1}$.mg protein$^{-1}$ (an increase of 82% above basal), the MTT conversion rate dropped from 1.798 ± 0.095 to 0.167 ± 0.003 U. mg protein$^{-1}$ ($P < 0.0001$) and the Rh123 fluoresence increased to 140 ± 8% ($P < 0.0001$) of the state IV membrane potential level (data not shown).

Observations made during MTT conversion studies suggested that significant mitochondrial swelling occurred in both liver and skeletal muscle mitochondria on the addition of concentrations of palmitate above 0.2 mM of palmitate. This swelling effect did not occur under state IV or state III respiratory conditions with either liver or muscle mitochondria in the absence of palmitate and did not occur when uncouplers such as FCCP or PCP (at concentrations that induced maximal rates of $\dot{V}O_2$) were added. Swelling assays as per Section 3.2 were not undertaken using fatty acids but when examined at an angle of 90° in respiratory buffer the mitochondria were seen to swell.

### 3.5.4 *Discussion*

Unlike succinate, fatty acids meet all the criteria established in the perfused hindlimb (Fig.'s 3.8 and 3.9) for an activator of muscle thermogenesis. In keeping with these results, fatty acids stimulated $\dot{V}O_2$, inhibited the MTT conversion rate and lowered the membrane potential in the isolated mitochondria.

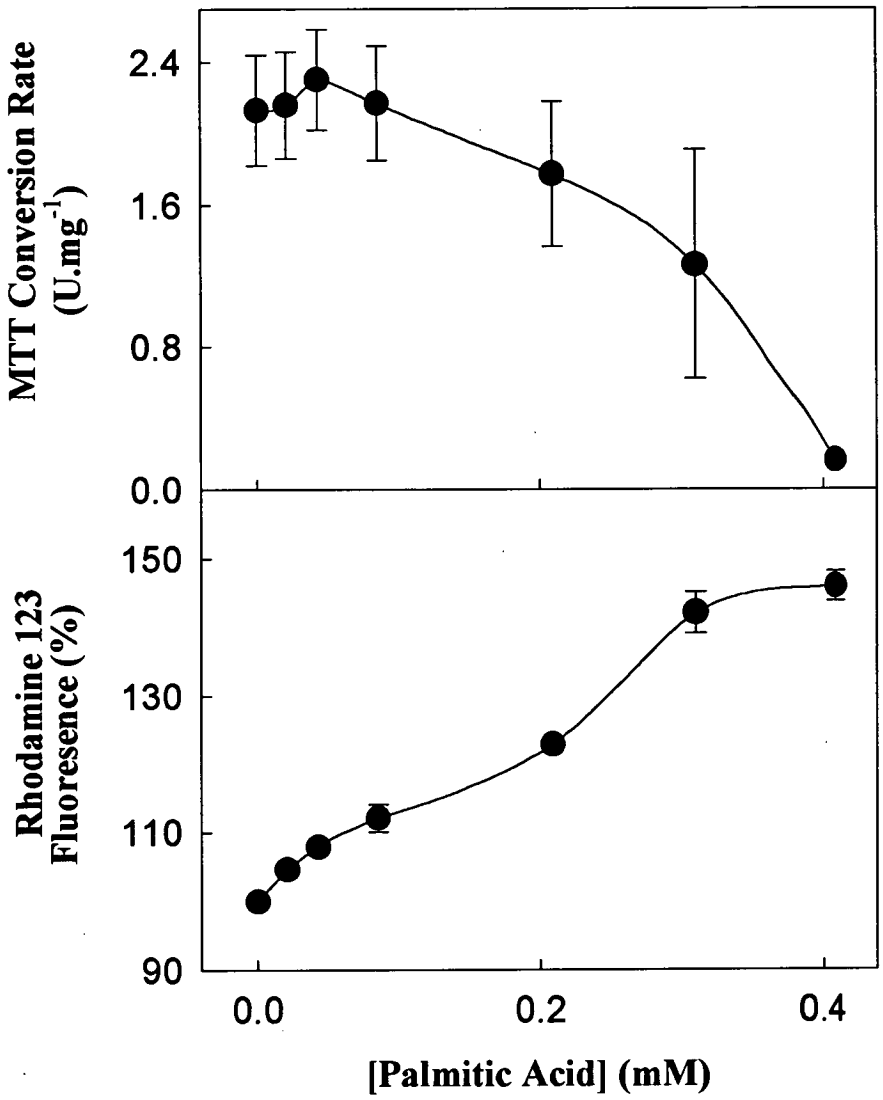


**Figure 3.15** MTT conversion rates and membrane potentials of SS skeletal muscle mitochondria in the presence of palmitate. State IV respiratory conditions were measured at 37°C, with pyruvate (5 mM) and malate (5 mM) added as the respiratory substrate. (n=4). $\dot{V}O_2$ increased from 22.18 ± 3.45 nmol.min$^{-1}$.mg protein$^{-1}$ by 7.80 ± 1.29 nmol.min$^{-1}$.mg protein$^{-1}$ on addition of 0.4 mM palmitate. This was calculated to be a significant increase using the paired two-tailed students t-test. Values are means ± S.E.M. When not visible, the error bars are within the symbol.

These fatty acid-induced changes in the isolated mitochondra were similar to those found on the addition of known uncouplers (FCCP or PCP) to mitochondria as discussed in Section 3.3.3 (and shown in Fig.'s 3.10, 3.11 and 3.12). These induced changes occurred in both liver and skeletal muscle mitochondria and the similarity of the results gained for all the measured parameters ($\dot{V}O_2$, MTT conversion and membrane potential) between known uncouplers and fatty acids suggested that they were both stimulating proton movement across the inner mitochondrial membrane. They are however using different mechanisms as shown by the fact that one (fatty acids) causes swelling of the respiring mitochondria while the other (uncouplers) does not.

Fatty acids are thus able to cause unregulated proton movement across the mitochondrial membrane but this effect is not tissue specific.

### 3.6 *General Discussion*

Examination of ATP, succinate and fatty acids as potential uncouplers of skeletal muscle mitochondria revealed that only fatty acids fulfilled all of the criteria established from studies in the perfused rat hindlimb.

ATP did not initiate a thermogenic process similar to that shown to be present in yeast mitochondria (Prieto *et al.*, 1992). Although ATP instigated swelling in muscle mitochondria similar to that reported for yeast mitochondria, no stimulation of $\dot{V}O_2$ was found to occur in the presence of atractylate and oligomycin.

Succinate caused maximal $\dot{V}O_2$ in the muscle mitochondria together with a decrease in the MTT conversion rate. Addition of ADP to the respiratory mix did not alter the rate at which oxygen was utilized, but the MTT conversion rate was increased back to the maximal level encountered when pyruvate + malate was the added substrate. These data were consistent with those from the perfused hindlimb on addition of NE, in that $\dot{V}O_2$ was high and the MTT conversion rate was inhibited compared to that seen under basal conditions. However, for isolated mitochondria the membrane potential was maximal and unaffected by succinate and contrasted with the perfused hindlimb on the addition of NE where the membrane potential was decreased. Thus, despite some similarities, succinate was deemed not to be the uncoupler affecting hindlimb $\dot{V}O_2$ and accounting for the stimulation due to NE.

Fatty acids affected the $\dot{V}O_2$, MTT conversion rate and membrane potential of the isolated mitochondria in a manner similar to that seen by the putative signal/substrate released by NE in the hindlimb, thus fulfilling all of the criteria established for such a candidate. As glycerol efflux occurs on addition of NE to the perfused rat hindlimb (Clark *et al.*, 1995; Clark *et al.*, 1994), the data strongly suggest that lipolysis is being stimulated and thus the possibility exists that fatty acids could be having an uncoupling action on the muscle mitochondria. This uncoupling action is not specific to muscle mitochondria, but appears to be a general mitochondrial response, indicating that all mitochondria and all tissues are able to respond to the thermogenic needs of an individual. The only limiting factor for a tissue's contribution to whole body thermogenesis would then be the number of mitochondria that it contains and the concentration of the respiratory enzymes that these mitochondria possess.

If fatty acid release is stimulated on addition of NE and other Type A vasoconstrictors to the perfused hindlimb, then the mechanism involved is novel. Normally, fatty acid release is via the same mechanism as described for BAT (Section1.3.3) involving a β-adrenergic mediated mechanism. In the perfused hindlimb β-agonists did not mimic the effect and β-antagonists did not inhibit the NE-

induced effect on both $\dot{V}O_2$ and glycerol release (Ye *et al.*, 1995). These observations together with hindlimb perfusion data suggesting that NE causes a redirection of perfusate flow, suggest that either the vasoconstriction or the redistribution of flow is responsible for the production of a paracrine substance (probably from or by the vasculature) that is able to stimulate triglyceride breakdown, fatty acid release and hence thermogenesis without the necessity for the stimulation of β-adrenoreceptors that are normally blocked by 10 μM DL propranolol.

# Chapter 4

## Final Discussion

### 4.1 *Summary of Findings*

The main aims of this thesis were to investigate the contribution of the vasculature to BAT thermogenesis and the possibility of a physiological uncoupler in skeletal muscle.
In this study:

(1) A technique for the isolation and constant flow perfusion of the periaortic BAT deposit was developed and characterized.

(2) BAT thermogenesis was found to be entirely β-mediated. The lack of an α-mediated or nitroprusside-sensitive component suggested that the vasculature did not contribute significantly either directly (e.g. "hot pipes") or indirectly (through alterations in blood flow) to BAT thermogenesis in the perfused preparation.

(3) ATP does not regulate a thermogenic process in SS skeletal muscle mitochondria similar to that shown to be present in yeast mitochondria (Prieto *et al.*, 1992).

(4) Although succinate increased $\dot{V}O_2$ and decreased the MTT conversion rate in SS skeletal muscle mitochondria, it did not decrease the mitochondrial membrane potential. It is therefore not likely to play an uncoupler role in the hindlimb muscle.

(5) Fatty acids were found to increase $\dot{V}O_2$ and decrease both the rate of MTT conversion and the mitochondrial membrane potential. Skeletal muscle may therefore possess an uncoupling mechanism with fatty acids as a likely candidate for the uncoupler. This mechanism would be similar to that found in BAT with the involvement of fatty acids but would not involve the uncoupling protein of BAT.

### 4.2 *Brown adipose tissue thermogenesis*

Thermogenesis in BAT does not appear to have a vascular component similar to that shown to be present in skeletal muscle. The stimulated thermogenesis in the perfused periaortic BAT preparation was entirely β-mediated as the response occurred both on the infusion of pure β-agonists (ISO and BRL 35135A) and on the infusion of the mixed α/β agonist (NE) in the presence of phentolamine (an α-antagonist). The use of these antagonists indicated that there was no direct α-adrenoreceptor mediated component contributing to the thermogenesis of the tissue, but lack of involvement of the vasculature was clearly indicated by both the absence of change in perfusion pressure on infusion of NE and the retention of the stimulated response in the presence of nitroprusside (a nitrovasodilator). In the perfused hindlimb, NE induces an increase in $\dot{V}O_2$ as well as perfusion pressure and both of these induced responses have been shown to be reversed by the addition of either α-antagonists or nitrovasodilators (Colquhoun *et al.*, 1988).

### 4.3 *Skeletal muscle thermogenesis*

If skeletal muscle does possess a mitochondrial uncoupler, then its action must be reversed with the onset of exercise. Figure 3.9 shows that the MTT conversion rate is returned to the basal level when muscle contraction is initiated despite NE still being present. This suggests that exercise either causes the production of a factor that recouples the mitochondria or stops the production or release of the factor stimulating uncoupling.

Of the potential uncouplers examined in this study, only fatty acids meet all the criteria established in the perfused hindlimb (Figs. 3.8 and 3.9). In accordance with this, when added to respiring, isolated mitochondria (both liver and subsarcolemmal skeletal muscle) fatty acids stimulated increases in $\dot{V}O_2$, and decreased both the MTT conversion rate and membrane potential.

For fatty acids to be able to act as physiological uncouplers, reversal of this process must be able to occur both easily and rapidly. ATP levels should not be compromised when the tissue is activated and function should not be impaired. In the skeletal muscle of an endotherm, lowered ATP levels could prevent full muscle contraction and thus interfere with the flight or fight response. If by involving skeletal muscle in the maintenance of a constant body temperature an organism renders itself vulnerable to a predator, then the advantages of homeothermy in a cold or changing environment would be negated.

During steady-state isometric contraction in the rat hindlimb *in vivo*, Brindle *et al.* (1989) showed that muscle concentrations of ADP, creatine and phosphate increase to 35 μM, 1.5 mM and 1.5 mM respectively. This makes any of these compounds good candidates for the role of recoupler of the fatty acid-induced mitochondrial uncoupling. Of these, ADP is perhaps the most likely as it is the mitochondrial matrix concentration of this that is directly responsible for the alteration of respiration from state IV to state III.

ADP has been shown by other researchers (Andreyev *et al.*, 1989; Mokhova *et al.*, 1993; Starkov *et al.*, 1994) to prevent the fatty acid-induced loss of membrane potential in both liver and muscle mitochondria. This is further evidence that the uncoupling induced in mitochondria by fatty acids only occurs under state IV respiratory conditions. With the switch of respiration from state IV to state III by the addition of ADP, normal oxidative phosphorylation should be able to occur, despite fatty acids still being present, as the mitochondria now possess the proton gradient necessary for ATP synthesis.

The uncoupling action of fatty acids and its seeming action under state IV but not state III respiratory conditions is analagous to the process known to occur in BAT mitochondria. In BAT, fatty acids interact with the uncoupling protein to instigate proton flux across the inner mitochondrial membrane from the intermembrane space to the matrix. This process has been shown (Nicholls and Locke, 1984) to be recoupled by purine nucleotides, of which ADP is one. The main difference between fatty acid-induced uncoupling in BAT mitochondria and those from liver and skeletal muscle, appears to be that the fatty acids are able to act as both the uncoupler and the fuel in BAT, but only as the uncoupler in the mitochondria from other sources as when added in the absence of other substrates (either succinate or pyruvate + malate) no increases in the respiratory rate are observed.

These data suggest that fatty acids are able to act as uncouplers of isolated mitochondria, but only under state IV or basal respiratory conditions, and that this uncoupling is similar to that seen in the presence of classical uncouplers (PCP and FCCP). In the hindlimb, it has been demonstrated (Fig.'s 3.8 and 3.9) that on stimulation of $\dot{V}O_2$, the MTT conversion rate and the membrane potential decrease in a manner similar to that noted in isolated mitochondria on the addition of both fatty acids and classical uncouplers. For fatty acids to be the instigator of uncoupling in the skeletal muscle of the perfused hindlimb on addition of certain vasoconstrictors, it would be necessary to examine the release of fatty acids by the perfused tissues under both basal and NE-stimulated conditions.

### 4.3.1 *Fatty acid release in perfused skeletal muscle*

Fatty acid release in the perfused rat hindlimb has been indirectly assessed by measurement of glycerol concentrations in the effluent perfusate. The assumption that glycerol release is indicative of fatty acid turnover has precedence (Ruderman *et al.*, 1971) and in the presence of NE not only are there increases in $\dot{V}O_2$ and perfusion pressure, but there is a marked rise in glycerol release from the perfused hindlimb (Clark *et al.*, 1995). This release of glycerol in conjunction with a stimulation of $\dot{V}O_2$ suggests that these two parameters might be linked and that the free fatty acids present in the skeletal muscle may be influencing mitochondrial respiration.

If fatty acids are the uncoupler acting in the perfused hindlimb on the addition of NE, then a β-mediated lipolytic component would be expected. This does not appear to be present, as in the presence of a broad β-antagonist (10 μM DL propranolol) NE is still able to induce increases in $\dot{V}O_2$ (Fig. 3.2) as well as in effluent glycerol concentrations. Furthermore, the stimulated increases in $\dot{V}O_2$ are reversed by nitrovasodilators and α-antagonists. The $\beta_1$, $\beta_2$, $\beta_3$-agonist isoproterenol fails to produce these changes and when added together with NE reverses the stimulated $\dot{V}O_2$ (Colquhoun *et al.*, 1990). Other vasoconstrictors (vasopressin and angiotensin II; Colquhoun *et al.*, 1988) have also been found to have a similar effect to that of NE when infused into the perfused rat hindlimb despite having no β-adrenoreceptor activity.

As suggested in Figs. 3.3 and 3.4 it appears that thermogenesis in skeletal muscle involves two distinct but necessary components. The first is the vasoconstriction-mediated redistribution of perfusate flow within the hindlimb and the second, is the production of an uncoupler substance, possibly fatty acids.

Vasoconstriction and the redistribution of perfusate flow is essential. NE, angiotensin II and vasopressin, each are able to cause vasoconstriction by direct interaction with receptors on the vasculature and if this component is prevented (e.g. by the use of nitrovasodilators) increases in $\dot{V}O_2$ do not occur.

The stimulation of triglyceride breakdown and the release of fatty acids may be more complex. As both angiotensin II and vasopressin do not have β-adrenoreceptor activity and yet are able to stimulate $\dot{V}O_2$ in a manner similar to that of NE, the involvement of β-adrenoreceptors in triglyceride breakdown becomes questionable. There are thus two major possibilities to explain the second component of the thermogenic process and how it is mediated (Fig. 4.1). The first is as suggested in Figure 3.4 (Hypothesis 3c in Section 3.1) where the vasoconstriction and/or the redistribution of flow is responsible for the production of a paracrine substance that is able to stimulate triglceride breakdown without the involvement of β-adrenoreceptors.

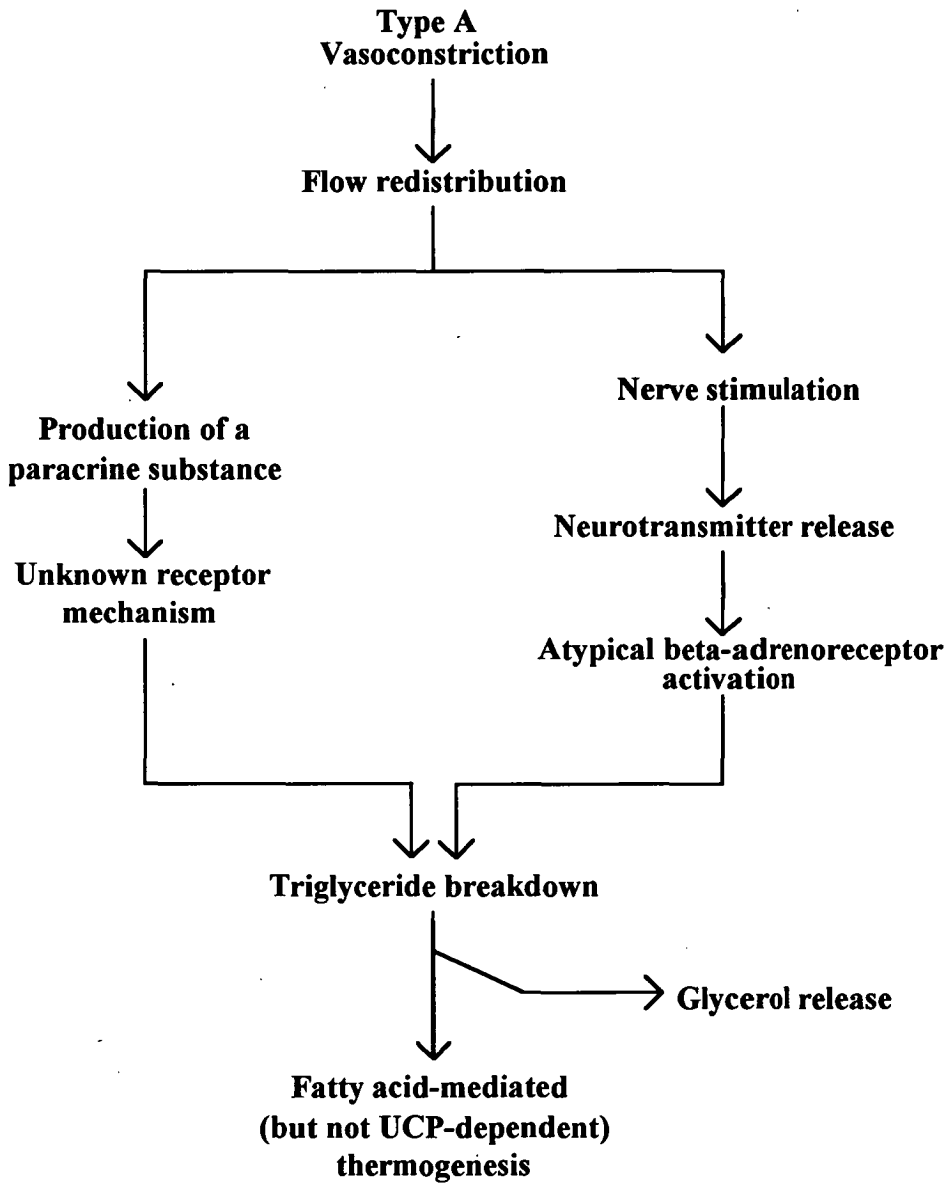


**Figure 4.1** Proposed mechanisms for the vasoconstrictor-mediated release of fatty acids and hence stimulation of thermogenesis. UCP = uncoupling protein.

The second possibility is that the vasoconstrictors are stimulating the release of neurotransmitters from the nerve terminals in the newly accessed areas that do have β-adrenoreceptor activity, and thus are able to stimulate triglyceride breakdown.

Recent work in rats *in vivo* (Carlisle and Stock, 1995) using a specific $\beta_3$-agonist (BRL 35135 A) has shown that the tissue glucose utilization index (GUI) response of BAT was inhibited by 1 $mg.ml^{-1}$ propranolol (estimated to be equivalent to 60 μM assuming a blood volume of 15 ml per 200 g rat) but the induced GUI in muscle remained unaffected at 20 $mg.ml^{-1}$ propranolol (approximately 1.2 mM). This could be suggestive of a population of atypical β-adrenergic receptors in muscle that are activated by BRL 35135A and are resistant to inhibition by propranolol but which mediate mechanisms similar to those classified as $\beta_3$ and present in BAT. As such, they could be responsible for the GUI (Carlisle and Stock, 1995) and fatty acid-dependent $\dot{V}O_2$. However, in Chapter 2 of this thesis, it was shown that the stimulation of $\dot{V}O_2$ by NE in the perfused BAT preparation was completely inhibited by 10 μM DL propranolol. This then suggests that to attain complete inhibition of an atypical β-adrenoreceptor ($\beta_3$ ?) mediated increase in $\dot{V}O_2$ in the perfused hindlimb would require concentrations of propranolol in excess of 200 μM. Thus for Type A mediated increases in $\dot{V}O_2$ a primary vasoconstriction and redistribution of blood flow is an essential prerequisite for a secondary release of catecholamine to interact with these putative atypical β receptors (Fig. 4.1).

### 4.3.2 *State IV involvement in thermogenesis*

Differences in metabolic rate between reptiles and mammals have been shown to contribute to the variations of body temperature encountered in these two phylogenetic groups. These differences in metabolic rate are maintained at the level of the isolated organ (Hulbert and Else, 1981; Else and Hulbert, 1987) and can be clearly demonstrated at the mitochondrial level (Else and Hulbert, 1981, 1985; Bennett, 1972; Hulbert and Else, 1989). These disparities in the respiration rate appear to be due not to differences in the surface area of the mitochondrial inner membrane, but to differences in the fatty acid composition of the mitochondrial phospholipids (Brand *et al.*, 1991).

Isolated mitochondria can respire at high rates without the neccessity for ATP synthesis due to a continual proton leak across the inner mitochondrial membrane that

largely controls (Hafner *et al.*, 1990) and accounts for virtually all of the oxygen consumed in the absence of ATP synthesis (Brand *et al.*, 1988). This respiration has been estimated to account for 20-40% of the basal rate of hepatocytes (Nobes *et al.*, 1990b; Brown *et al.*, 1990).

This state IV proton leak decreases with increasing body mass in mammals (Porter and Brand, 1993). As larger mammals have a lesser surface to mass ratio than do smaller ones and thus a smaller heat loss to the environment, this decrease in mitochondrial proton leak would cause less heat production. The proton leak is regulated by phosphorylation of ADP. Brand *et al.* (1993) showed that as ATP turnover and substrate oxidation increased proton leak decreased.

Mitochondrial proton leak in most if not all of an endotherms tissues appears likely as a means of thermogenesis. With regulation of the rate of proton leak by the production, presence and hence neccessity for rephosphorylation of ADP, this thermogenic mechanism would not interfere with normal tissue functions. Fatty acids seem to reinforce the coupling defect (or proton leak) in mitochondria (Barre *et al.*, 1989) and hence contribute to the thermogenic output of the mitochondria and the tissue.

Fatty acid-induced increases in the rate of proton leak under state IV or low state III respiratory conditions seems a viable candidate for a thermogenic process in skeletal muscle. The mechanism is similar to that seen in BAT, especially as these processes are completely inhibited in the presence of high concentrations of ADP (*i.e.* high state III respiratory conditions produced with the onset of exercise).

### 4.4 *Conclusions*

Thermogenesis in BAT does not appear to have a significant vascular component similar to that found in skeletal muscle. However, fatty acids are candidates for an uncoupler effect in skeletal muscle. Vasoconstrictors seem able to control thermogenesis by both the regulation of tissue blood flow and the production of fatty acids which are able to increase the rate of mitochondrial proton leak under state IV respiratory conditions. This thermogenic mechanism appears to be common to all tissues and all homeothermic species and may be a general physiological mechanism for the maintenance of homeothermy. The kidney, intestine and mesenteric artery network from the rat exhibit increases in both perfusion pressure and $\dot{V}O_2$ in response to the infusion of vasoconstrictors to the perfused tissues (Ye *et al.*, 1990b), and similar responses have been shown to occur in the perfused hindlimb of chickens (Appendix 1) and the marsupial rat-kangaroo *Bettongia gaimardi* (Ye *et al.*, 1995).

## 4.5 *Future considerations*

In order to further link cold-induced mitochondrial leakiness and fatty acid-induced uncoupling, two factors that could be involved with thermogenesis, it would be necessary to assess proton leak in muscle mitochondria. This has been shown to occur in liver mitochondria, but proper assessment of its presence and contribution to skeletal muscle thermogenesis is essential.

All three parameters ($\dot{V}O_2$, MTT conversion rates and membrane potential) should be examined in animals raised in either thermoneutral (25°C) or cold (4°C) environments, and a comparison of state IV and state III conditions made for the mitochondria in the presence and absence of fatty acids. Measurement of different rates of ADP phosphorylation by the mitochondria could also contribute to an understanding of thermogenesis and endothermy. The investigation of mitochondria from different endotherms (mammals, birds and marsupials) would allow assessment of this process (both proton leak and fatty acid stimulation of this process) as a widespread physiological thermogenic process.

The effect of fatty acids in the perfused hindlimb should also be examined but for accurate assessment of the effective fatty acid concentrations this would require perfusion in the absence of albumin. Albumin binds fatty acids thus preventing delivery, but is necessary to maintain oncotic pressure in the perfused system. Removal of albumin from the perfusate will lead to rapid tissue odema, and thus a suitable substitute must be used. Further examination of the possibility of β-adrenoreceptor mediated induction of triglyceride breakdown is also needed. This would include the use of higher concentrations of propranolol, the prevention of neurotransmitter release and the examination and comparison of neurotransmitter concentrations in both the muscles and effluent perfusate under basal and stimulated conditions.

Examination of the MTT conversion rate and [3H]-TPMP uptake under both basal and NE-stimulated respiratory conditions in the perfused BAT preparation would be useful in the classification of the hindlimb NE-induced response as either coupled or uncoupled respiration.

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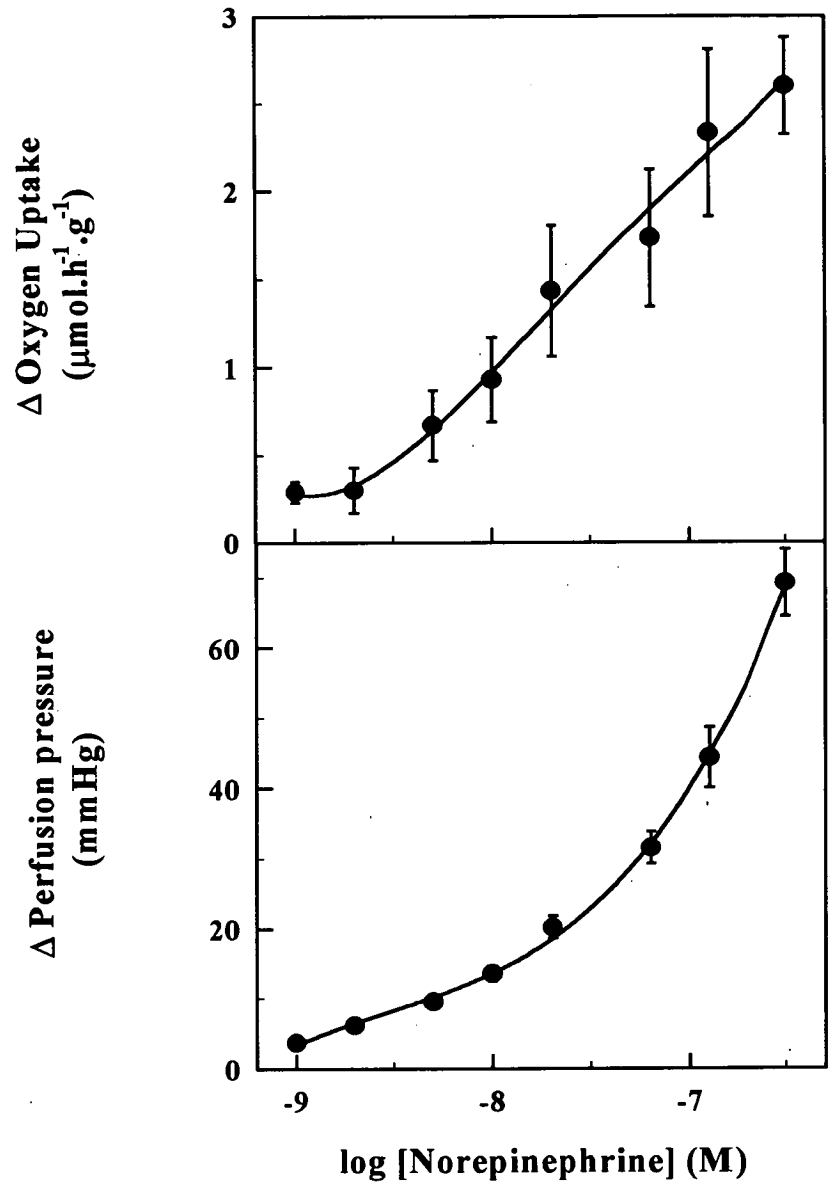
# Appendix 1




Dose response curve for norepinephrine on oxygen uptake and perfusion pressure in the perfused chicken lower limb at 25°C using a Krebs-ringer bicarbonate buffer as described in Section 2.2.1 (but with 2% BSA) with a flow rate of 5 $ml.min^{-1}$. 15 g $muscle^{-1}$. Values are means ± S.E.M. When not visible the error bars are within the symbol. (n > 4) (T.P.D. Eldershaw, C.Duchamp, M.G. Clark and E.Q. Colquhoun, personal communication, 1994).

# Appendix 2

## *Publication directly arising from this thesis*

MATTHIAS, A., RICHARDS, S.M., DORA, K.A., CLARK, M.G. and COLQUHOUN, E.Q. 1994. Characterization of perfused periaortic brown adipose tissue from the rat. *Can. J. Physiol. Pharmacol.* **72**: 344-352.

## *Book monograph*

CLARK, M.G., COLQUHOUN, E.Q., DORA, K.A., RATTIGAN, S., ELDERSHAW, T.P.D., HALL, J.L., MATTHIAS, A. and YE, J-M. 1994. Resting muscle: A source of thermogenesis controlled by vasomodulators. In *Temperature Regulation - Advances in Pharmacological Sciences. Edited by* A.S. Milton. Birkhauser, Verlag, Basel. pp. 315-320.

## *Papers presented at scientific meetings*

CLARK, M.G., MATTHIAS, A., DORA, K.A., RATTIGAN, S., ELDERSHAW, T.P.D., HALL, J.L. and COLQUHOUN, E.Q. 1993. Vascular control of muscle metabolism: possible novel thermogenic mechanisms regulated by vasomodulators. *Proc. Aust. Soc. Biochem. Mol. Biol. (WA branch).*

MATTHIAS, A., COLQUHOUN, E.Q., RICHARDS, S.M. and CLARK, M.G. 1991. Perfusion of hyperplastic periaortic brown adipose tissue. *Proc. Aust. Soc. Biochem. Mol. Biol.* **23**: SP48.

MATTHIAS, A., COLQUHOUN, E.Q. and CLARK, M.G. 1993. Unusual properties of skeletal muscle subsarcolemmal mitochondria with thermogenic potential. *Proc. Aust. Soc. Biochem. Mol. Biol.* **25**: COL-6-2.

MATTHIAS, A., DORA, K.A., RATTIGAN, S., YE, J-M., COLQUHOUN, E.Q. and CLARK, M.G. 1994. Comparison of thermogenesis in perfused skeletal muscle and brown adipose tissue. *Modern Trends in BioThermoKinetics.* **3**: 249-250.

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